REMARKS

At the outset, Applicants would like to thank the Examiner for the courtesy of the personal Examiner's Interview conducted with Applicants' representatives on January 7, 2004, during which the claims in the above-referenced application were discussed.

Applicants note that the Amendments filed May 5, 2003 and August 7, 2003 have been entered and that the Formal Drawings submitted on May 5, 2003 were found to be in compliance with 37 C.F.R. § 1.84.

Claims 50, 53, and 57-58 were pending in the above-referenced application.

I. Amendments to the Claims

Claims 50, 53 and 57-58 have been canceled without prejudice or disclaimer of the subject matter contained therein. Applicants reserve the right to pursue the subject matter of the canceled claims in this or future related applications.

Claims 60-77 have been newly added. Support for the newly added claims can be found throughout the specification as filed. Specifically, support for the newly added claims can be found at page 2, ll. 9-38 continuing on page 3, ll. 1-10 and ll. 15-17; page 7, ll. 2-6; page 8, ll. 12-20; page 9, ll. 5-8; page 16, ll. 20-22; p.21, ll. 3-9; p.22, ll. 7-14; page 23, ll. 4-9; page 24, ll. 4-11 and ll. 22-36; page 33, ll. 7-14; and Example 17. It is submitted that no new matter has been added.

II. Rejections under 35 U.S.C. § 102(e)

Applicants gratefully acknowledge that the previous rejection of the claims under 35 U.S.C. § 102(e), for allegedly being anticipated by Ledbetter et al. (U.S. Patent No. 6,010,902), has been withdrawn, because the Examiner deemed the referenced

heteroconjugates or bispecific antibodies to be distinguishable from the claimed antibodies covalently attached to a surface (Office Action, page 2, paragraph 4).

III. Rejections under 35 U.S.C. § 103

Claims 50, 53 and 57-58 stand rejected under 35 U.S.C. § 103 as being unpatentable over Ledbetter et al. (EP0440373) in view of Ledbetter et al. (U.S. Patent No.: 6,010,902) and Chang (U.S. Patent No.: 6,129,916) (Office Action, page 2, paragraph 5).

With the cancellation of claims 50, 53 and 57-58, this rejection as it applies to these claims has been rendered moot. Thus, Applicants address this rejection as it may potentially apply to newly added claims 60-77.

The Examiner relies on Ledbetter et al. (EP0440373) to teach "methods of activating T lymphocytes with immobilized anti-CD3 and immobilized anti-CD28 antibodies." The Examiner admits that "Ledbetter et al. differ from the claimed methods by not exemplifying combining anti-CD28 and anti-CD3 antibodies on the same plate," but alleges that "Ledbetter et al. do teach combining both specificities to stimulate T cells and to immobilize both antibodies on plastic surfaces" (Office Action, page 3, first and second paragraphs).

The Examiner relies on Ledbetter et al. (U.S. Patent No.: 6,010,902) to teach "stimulating T cells with the combination of antibodies to CD3 and anti-CD28 antibodies (e.g. 9.3) in order to stimulate T cell populations and subpopulations and reinfused in patients (e.g. see columns 15-16) (see entire document, including Detailed Description of the Invention and Examples)" and for teaching that "these cell populations have increased signal transduction, which can be measured by various known assays" (Office Action, page 3, last paragraph).

The Examiner relies on Chang (U.S. Patent No.: 6,129,916) to teach "combining the particular CD3 and CD28 specificities, by teaching the use of microbeads and cross-linking by well-established manner (columns 7-8) in cross-linking anti-CD3 and anti-CD28 antibodies on microbeads to activate T cells *in vivo*" (Office Action, page 4, third paragraph). The Examiner alleges that although Chang focuses on the *in vivo* administration of stimulating immunoconjugates, "it was known to stimulate T cells *in vitro* via immobilized stimuli" and further, that "both Ledbetter et al. references teach stimulating T cells for adoptive immunotherapy via CD3 and CD28 stimulation" (Office Action, page 4, fourth paragraph).

Finally, the Examiner alleges that "it was an art known practice to monitor cell proliferation of interest, including cell size and cell markers at the time the invention was made; as such criteria were known parameters of cell activation." The Examiner also opines that "it was common practice at the time the invention was made to re-activate and re-stimulate cells to maintain proliferation and expansion of cell populations of interest a the time the invention was made" (Office Action, page 4, last paragraph).

Applicants respectfully traverse the Examiner's assertion that the claimed invention would have been obvious to the skilled artisan at the time the invention was made. Reconsideration and withdrawal of this rejection in light of the following arguments is respectfully requested.

Newly added claim 60, and claims depending therefrom, are directed to methods for inducing *ex vivo* proliferation of a population of T cells ***to sufficient numbers for use in therapy*** by contacting said T cells with anti-CD3 and anti-CD28 antibodies ***covalently attached to the same surface*** thereby inducing the population of T cells to proliferate to sufficient numbers for use in therapy.

To establish a *prima facie* case of obviousness, the Examiner has the burden of showing either that some objective teaching in the prior art or knowledge generally available to one of ordinary skill in the art would have motivated that individual to combine the relevant teachings of the references to arrive at the claimed invention. *In re Fine*, 5 USPQ2d 1596, 1598 (Fed. Cir. 1988). Indeed, the prior art must suggest the combination or convey to those of ordinary skill in the art a reasonable expectation of success of making it. *In re Vaeck*, 947 F.2d 488, 20 USPQ2d 1438 (Fed. Cir. 1991). The teachings of the references can be combined only if there is some suggestion or incentive to do so. *ACS Hospital Systems, Inc. v. Montefiore Hospital*, 221 USPQ 929, 933 (Fed. Cir. 1984). Under § 103, both the suggestion and the expectation of success must be founded in the prior art, not in applicant's disclosure. *Amgen, Inc. v. Chugai Pharmaceutical Co., Ltd.* 927 F.2d 1200, 1207, 18 USPQ2d 1016 (Fed. Cir. 1991). Moreover, when a combination of references are used to establish a *prima facie* case of obviousness, the Examiner must present evidence that one of ordinary skill in the art would have been motivated to combine the teachings in the applied references in the proposed manner to arrive at the claimed invention. *Carella v. Starlight Archery*, 804 F.2d 135, 231 USPQ 644 (Fed. Cir. 1986).

With respect to Ledbetter et al. (EP0440373), Applicants respectfully submit that nowhere in this reference is there a teaching or suggestion to covalently attach anti-CD3 antibody or fragments thereof ***and*** anti-CD28 antibody or fragments thereof on the ***same*** surface to induce *ex vivo* proliferation of a population of T cells to sufficient numbers for use in therapy. Moreover, when ***taken as a whole***, this reference teaches away from the claimed invention, as it is directed to "find a novel and efficient method of stimulating T cells to develop ***CD3-independent*** cytolytic activity to attack tumor cells" (see, page 2, ll. 34-35). Thus, one of skill in the art, based on the teachings of this reference would not have been motivated to use a combination of anti-CD3 and anti-

CD28 antibodies, much less immobilize them on the same surface to induce *ex vivo* proliferation of a population of T cells to sufficient numbers for use in therapy.

Ledbetter et al. (U.S. Patent No.: 6,010,902) do not remedy the deficiencies of the primary reference. Instead, this reference amplifies this deficiency by teaching away from combining anti-CD3 and anti-CD28 antibodies to stimulate T cells. Specifically, Ledbetter et al. ('902) disclose that CD3/CD28 heteroconjugates comprising monoclonal antibodies G19-4 and 9.3 (column 24, lines 60-62) do not show any significant increase in its ability to activate T cells (as measured by intracellular calcium mobilization) over unstimulated or CD3/CD3-stimulated cells (see column 24, lines 63-67 and column 25, lines 1-34). This is in ***striking contrast*** to the CD3/CD2, CD3/CD4, CD3/CD6 and CD3/CD8 heteroconjugates, ***all of which*** induce a marked increase in calcium mobilization within the T cells treated with those heteroconjugates as compared to the activity of unstimulated or CD3/CD3-stimulated cells (column 25, Table III and lines 25-30). Thus, Ledbetter et al. ***teaches away*** from using anti-CD3 and anti-CD28 to induce T cell proliferation, since the heteroconjugate comprised of anti-CD3 and anti-CD28 antibodies does not stimulate T cell activation over background levels compared to heteroconjugates comprised of other T cell surface marker combinations which support robust activation. Furthermore, even if the CD3/CD28 heteroconjugates or bispecific antibodies of Ledbetter et al. did stimulate T cell activation (which is denied), this would still not render obvious Applicants' claimed invention, because heteroconjugates or bispecific antibodies are significantly different to antibodies covalently attached to the same surface. Thus, in contrast to the Examiner's assertions, Ledbetter et al. ('902) do not teach stimulating T cells with combination of anti-CD3 and anti-CD28 antibodies, but rather teaches away from using this combination.

Chang (U.S. Patent No.: 6,129,916) also does not remedy the deficiencies of the references discussed above. First, Chang teaches away from *in vitro* methods of activating T cells by teaching that a major concern with ***in vitro*** regimens

> is that the treatment is very tedious, expensive, and requires a sophisticated, specialized cell culture facility. The variation among cells or cultures from different patients requires demanding monitoring procedures. Also, ***lymphocyte cultures have very poor viability even under optimal conditions, meaning that during the culturing, large numbers of the cells will die.*** When large numbers of dead cells are injected into patients, this may actually burden the reticuloendothelial system (RES) and reduce its effectiveness in combating the tumor cells. [***emphasis added***]. See column 3, lines 19-27.

In view of the foregoing teaching by Chang, one of ordinary skill in the art would not have been motivated to activate T cells *in vitro* with the Chang conjugates to induce proliferation of a population of T cells to sufficient numbers for use in therapy as proposed by the Examiner.

Chang discloses immunoregulatory conjugates including a polymeric backbone coupled with binding molecules, for example, antibodies which bind to monovalent antigenic epitopes on CD3, epitopes of the T cell receptor, or other antigens on the surface of T cells, e.g., CD2, CD4, CD5, CD8, or CD28 (col. 4, ll. 39-52) or antibodies specific for HLA class I antigens, HLA class II antigens, or anti-CD37 (col. 11, ll. 32-36) for use in activating T cells *in vivo*. There is no teaching or suggestion in Chang that would motivate an ordinary skilled artisan to select the antibodies that bind CD3 and antibodies that bind CD28 among the list of antibodies, which are taught to be equally useful for activating T cells *in vivo*. In fact, based on the teachings of Ledbetter et al. ('902) that CD3/CD28 is ineffective in activating T cells compared to other T cell surface marker combinations (*see*, discussion above), there simply would be no motivation for one of ordinary skill in the art to choose anti-CD3 and CD28 antibodies out of the laundry list of antibodies to T cell surface markers listed in Chang. Additionally, in

light of the data discussed during the Examiner's Interview of January 7, 2004, which showed that T cell proliferation is dramatically different depending on which combination of antibodies are coupled to the beads that are used to stimulate the T cells (*see*, Appendix A), Applicants respectfully submit that Chang simply did not appreciate these unexpected differences between the different combinations of antibodies in his wish list of antibodies. Chang provides no distinction whatsoever between the antibody combinations he lists with regard to T cell activation.

Thus, Applicants respectfully aver that the proposed combination of references fails to teach or suggest the invention as claimed in the currently pending claims. Moreover, although not acquiescing that the Examiner has established a *prima facie* case of obviousness, as discussed at the January 7, 2004 Examiner's interview, there is abundant evidence of secondary considerations to support the non-obviousness of the claimed invention.

First, the non-obviousness of the invention is apparent from the results achieved when the invention is put into practice. More specifically, use of the claimed invention as described in the specification, allows for the long-term growth and proliferation of T cells. As demonstrated by Levine, B.L. et al. (1997) *J. Immunol.* **159**:5921-5930 (Appendix B) and Levine, B. L. et al. *Science* **272**(5720):1939-43 (Appendix C) submitted herewith, T cells stimulated with anti-CD3 and anti-CD28 antibodies attached to the same surface pursuant to the teachings of the present invention, remained in exponential proliferation for between 35 to 50 days in culture (Figures 1A and 2A in Appendix B and Fig. 1A in Appendix C). As discussed during the Examiner's Interview, both soluble anti-CD28 antibodies combined with immobilized anti-CD3 antibodies and anti-CD3 and anti-CD28 antibodies attached on separate surfaces are far less efficient, in stimulating exponential T cell proliferation than anti-CD3 and anti-CD28 antibodies attached on the same surface.

In addition, as discussed during the Examiner's Interview, Applicants have demonstrated that T cells treated *ex vivo* with anti-CD3 and anti-CD28 antibodies attached to the same surface as described in Applicants' specification, exhibit increased cell growth and viability over time as compared to T cells treated using other protocols. A good surrogate for T cell proliferation, and an important property reflecting clinical utility is the survival of the infused T cells in patients after *ex vivo* growth. In independent trials, which were discussed at the Examiner's Interview, it was found that T cells treated as taught in Applicants' specification, had high levels of survival of the infused T cells (for example, up to 48 weeks post-infusion compared to 12 weeks of previous approaches). Also, T cells activated as taught by Applicants' specification also maintain the T cell repertoire of the starting T cell population unlike other T cell activation protocols. Additionally, such T cells treated have roughly the same post-thaw viability as pre-freeze viability; this property is especially important when T cells have to be frozen from patients for later use, for instance after chemotherapy.

Furthermore, as discussed with the Examiner during the interview, the claimed invention has received professional approval and achieved commercial and clinical success. In fact, the work reported in the specification and claimed herein has been published in the prestigious journal "Science" (*see*, Appendix C). The work reported in the specification has also been commercialized (the patent application has been licensed to Xcyte Therapies, Inc., Seattle, WA) and is currently being utilized in Phase I and Phase II clinical trials. As discussed at the Examiner's Interview, these clinical trials have shown promising early results. The T cells infused after *ex vivo* activation as claimed herein led to rapid lymphocyte recovery in multiple myeloma and prostate cancer clinical trials. In the multiple myeloma trials, the TCR Vβ skewing seen in multiple myeloma patients was reduced upon infusion of the T cells activated by the method taught in Applicants' specification and the TCR repertoire was normalized.

The T cells produced by the claimed method have also been shown to reduce bone metastases three months after treatment in some patients. Also, in chronic lymphocytic leukemia trials, the enlarged spleen and lymph nodes of patients were reduced in size following infusion of the T cells expanded *ex vivo* as taught by Applicants. In addition, Applicants' methods have also been found to be of potential use in treating head and neck squamous cell carcinomas.

Finally, the significance of Applicants teachings are also to be found in the numerous instances of others using the methods taught by Applicants' specification to stimulate T cells for use in therapy or (Appendix D provides a few examples: Hellstrom et al. (2001), *Proc. Natl. Acad. Sci. USA* **98**(12):6783-88; Orchard, P.J. et al. (2002) *Hum Gene Ther.* **13**(8):979-88; and Pene J. et al. (2003) *J. Immunol. Methods* **283**:59-66)

For the foregoing reasons, Applicants respectfully request that the rejection under 35 U.S.C. § 103 be reconsidered and withdrawn.

III. Provisional Rejection under the doctrine of obviousness-type double patenting

Claims 50, 53 and 57-58 stand provisionally rejected under the doctrine of obviousness-type double patenting as being unpatentable over copending claims of USSN 08/253,964, USSN 08/592,711, USSN 09/183,055, USSN 09/349,915; and USSN 09/553,865 (Office Action, page 5, paragraph 6).

The Examiner is requested to clarify why a provisional double patenting rejection has been applied over USSN 09/183,055, which is clearly patentably distinct from the claimed invention.

While in no way admitting that claims 50, 53 and 57-58 are obvious over the claims of the other co-pending applications listed above, upon allowance of the claims of these co-pending applications, Applicants will consider submitting a Terminal

Disclaimer in compliance with 37 C.F.R. § 1.321(b) and (c), if appropriate, which will obviate this rejection.

IV. Rejection under the doctrine of obviousness-type double patenting

Claims 50, 53 and 57-58 stand rejected under the doctrine of obviousness-type double patenting as being unpatentable over claims 1-32 of U.S. Patent No. 6,352,694 (Office Action, page 6, paragraph 8) and over claims 1-29 of U.S. Patent No. 6,534,055 (Office Action, page 6, paragraph 9).

While in no way admitting that claims 50, 53 and 57-58 are obvious over the claims of U.S. Patent No. 6,352,694 and U.S. Patent No. 6,534,055, upon allowance of the claims of the instant application, Applicants will consider submitting a Terminal Disclaimer in compliance with 37 C.F.R. § 1.321(b) and (c), if appropriate, which will obviate this rejection.

V. Conclusion

Applicants believe that all of the outstanding rejections of record have been overcome by amendment and/or argument. Accordingly, the claims are now believed to be in condition for allowance. Applicants respectfully request that the Examiner issue a timely Notice of Allowance.

No fees are believed to be due in connection with this correspondence. If any fees are due, please charge any payments due, or credit any overpayments, to our Deposit Account No. 08-0219.

The Examiner is invited to telephone the undersigned at the telephone number given below in order to expedite the prosecution of the instant application.

Respectfully submitted,

Reg. No. 33,523
for Colleen Superko

Dated: January 15, 2004

Colleen Superko
Reg. No. 39,850

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APPENDIX A

Data discussed at the Examiner interview showing the unexpected properties of anti-CD3 and anti-CD28 attached on the same surface compared to anti-CD3 in combination with antibodies to other T cell surface markers.

Day 4 PC063

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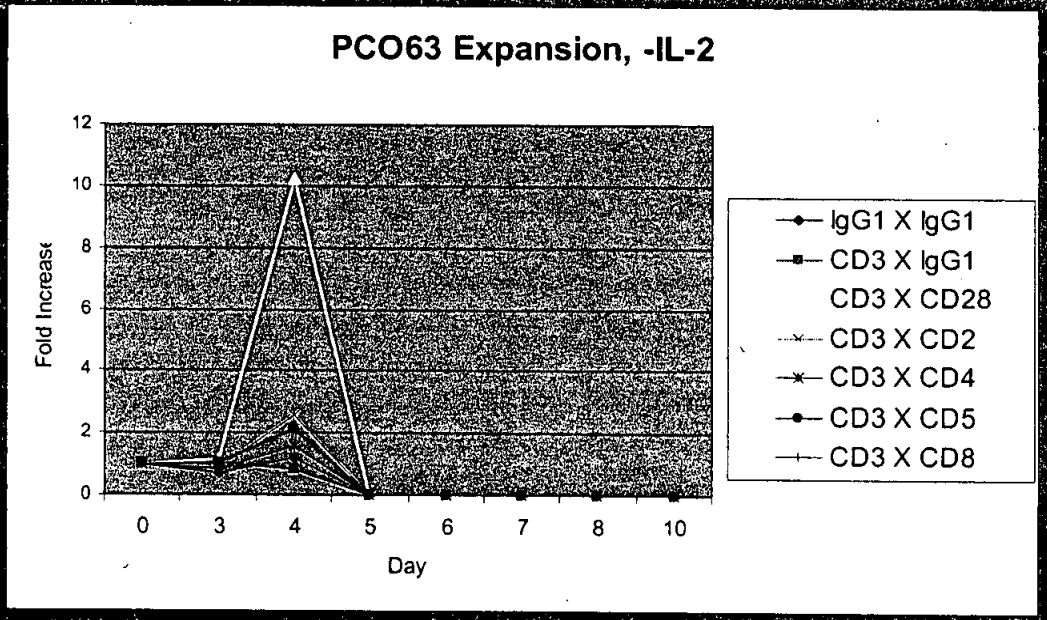

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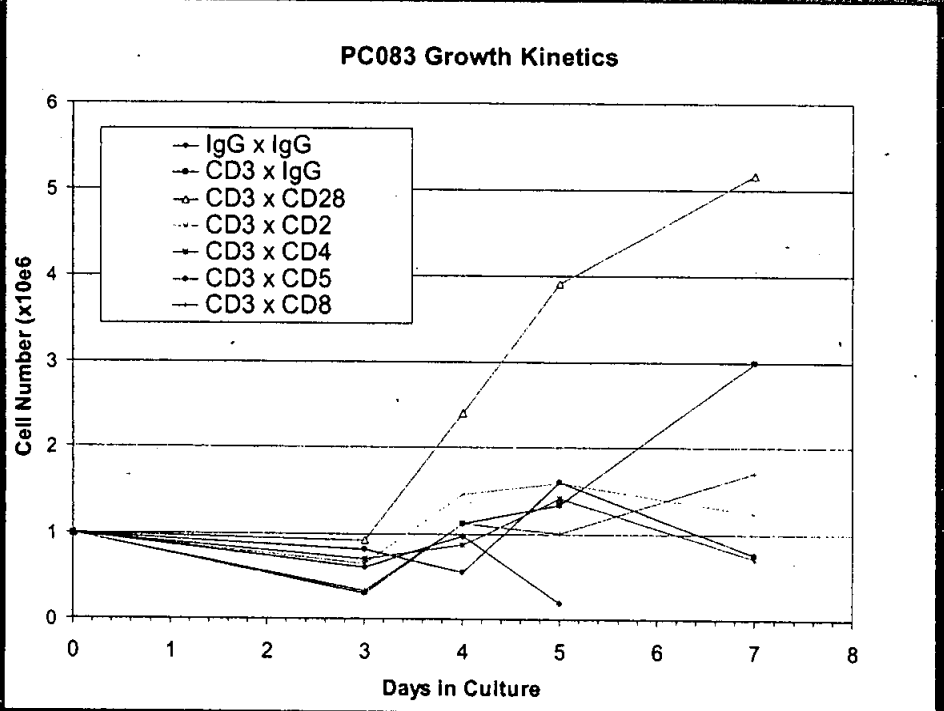

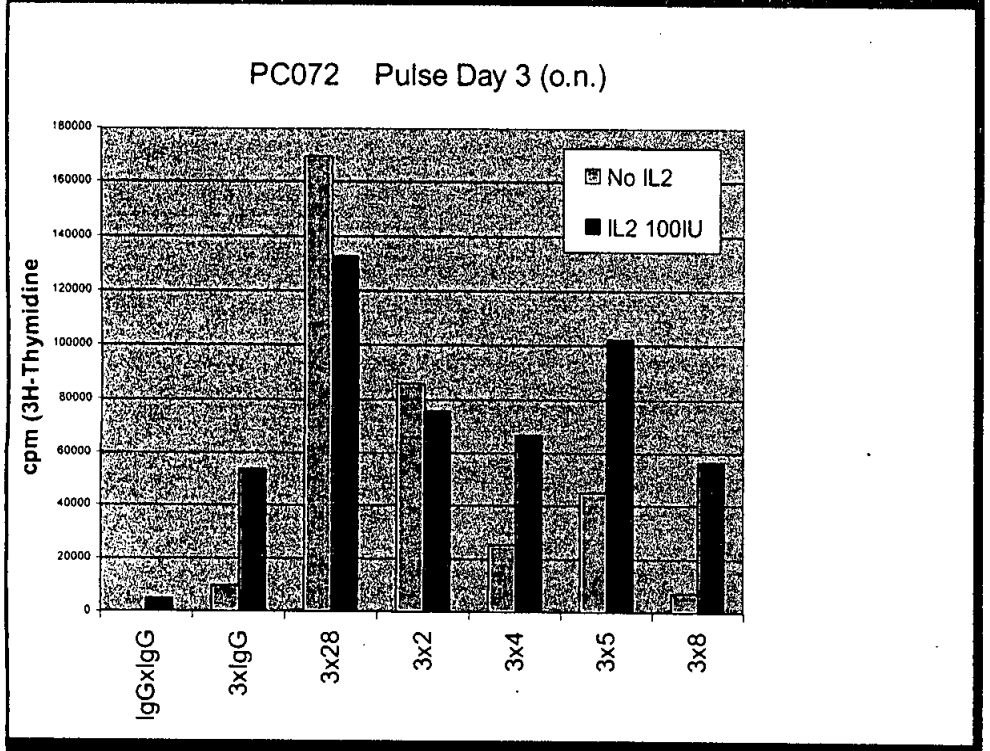
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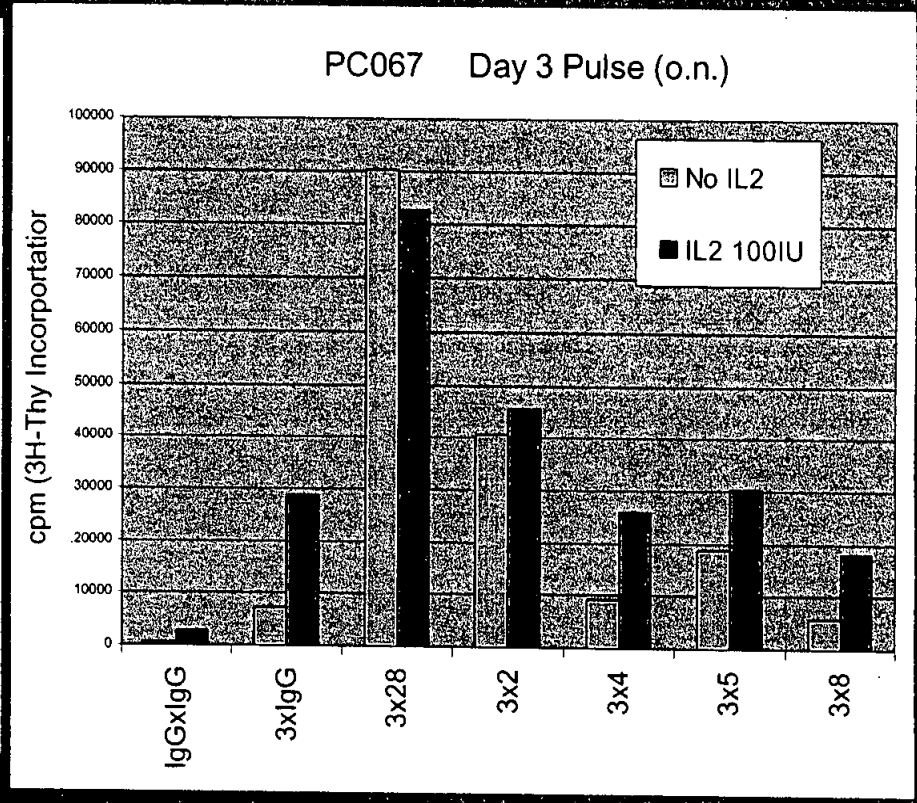
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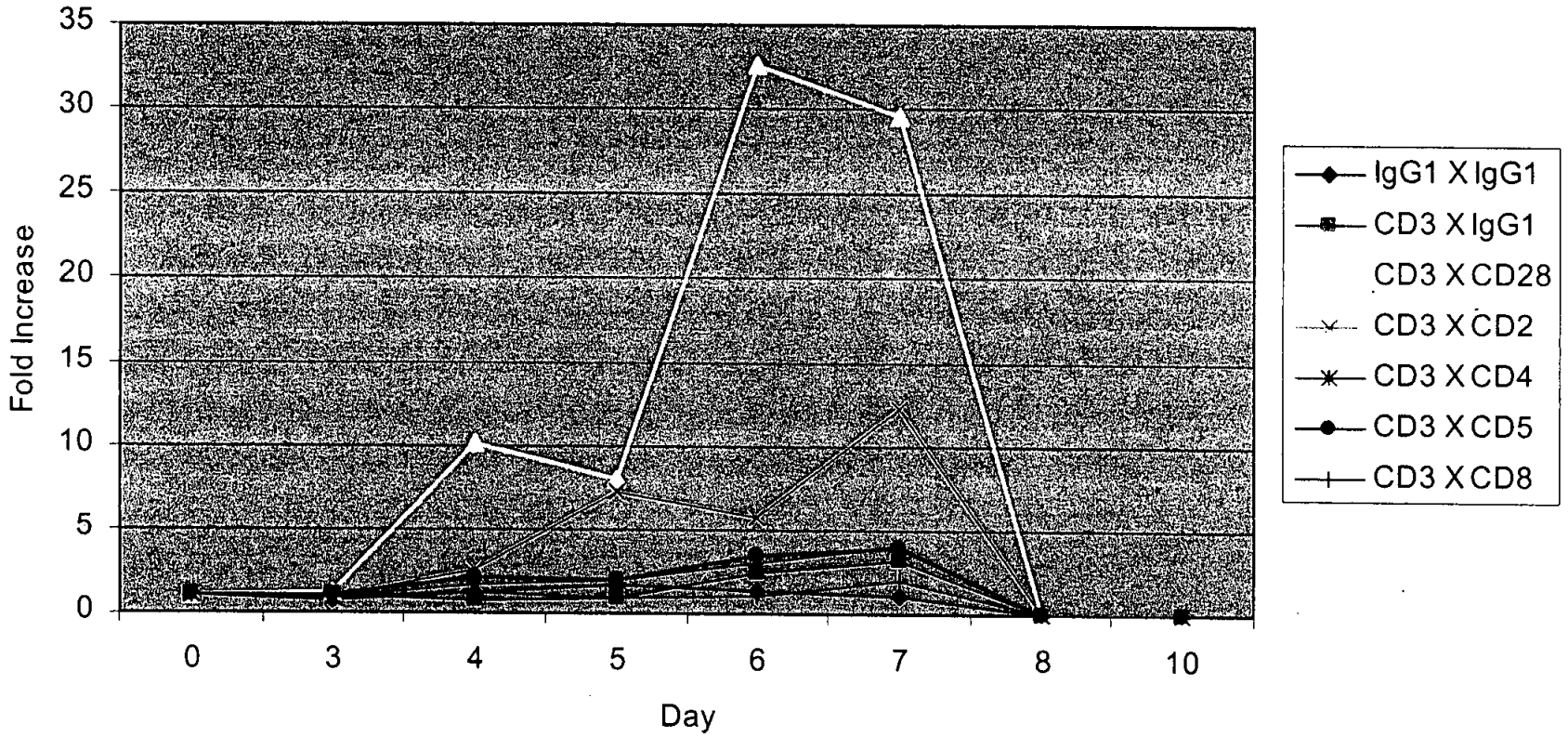

APPENDIX B

Levine, B.L. et al. (1997) *J. Immunol.* **159**:5921-5930

Effects of CD28 Costimulation on Long-Term Proliferation of CD4$^+$ T Cells in the Absence of Exogenous Feeder Cells[1]

Bruce L. Levine,[2*] Wendy B. Bernstein,[†] Mark Connors,[‡] Nancy Craighead,[§] Tullia Lindsten,[¶] Craig B. Thompson,[¶] and Carl H. June*

In this report, conditions for prolonged in vitro proliferation of polyclonal adult CD4$^+$ T cells via stimulation with immobilized anti-CD3 plus anti-CD28 have been established. CD4$^+$ cells maintained exponential growth for more than 60 days during which a total 10^9- to 10^{11}-fold expansion occurred. Cell cultures exhibited cyclical changes in cell volume, indicating that, in terms of proliferative rate, cells do not have to rest before restimulation. Indeed, electronic cell size analysis was the most reliable method to determine when to restimulate with additional immobilized mAb. The initial ~10^5-fold expansion was autocrine, occurring in the absence of exogenous cytokines or feeder cells. Addition of recombinant human IL-2 after the initial autocrine expansion resulted in continued exponential proliferation. Phorbol ester plus ionomycin also induced long-term growth when combined with anti-CD28 stimulation. Analysis of the T cell repertoire after prolonged expansion revealed a diverse repertoire as assessed by anti-TCR Vβ Abs or a PCR-based assay. Cytokines produced were consistent with maintenance of both Th1 and Th2 phenotypes; however, the mode of CD3 and CD28 stimulation could influence the cytokine secretion pattern. When anti-CD3 and anti-CD28 were immobilized on the same surface, ELISAs on culture supernatants revealed a pattern consistent with Th1 secretion. Northern analysis revealed that cytokine gene expression remained inducible. Spontaneous growth or cell transformation was not observed in more than 100 experiments. Together, these observations may have implications for gene therapy and adoptive immunotherapy. Furthermore, these culture conditions establish a model to study the finite lifespan of mature T lymphocytes. *The Journal of Immunology,* **1997, 159: 5921–5930.**

In vivo, T lymphocytes require at least two signals for complete activation. In addition to an Ag signal, a second costimulatory signal allows a T helper cell to produce sufficient IL-2 and other cytokines to allow autocrine-driven clonal expansion (1, 2). The CD28 receptor on T cells is able to provide such a costimulatory signal following interaction with CD80 or CD86 on APCs (2–4). CD28 stimulation has been shown to stabilize cytokine mRNA and can also induce the cell survival gene Bcl-X_l (5). Indeed, the antiapoptotic effect appears to be a major role for CD28 ligation in vivo (6). Recently, the CCR1, CCR2, and CCR5 β chemokine receptors have been identified as the first described genes that are down-regulated following CD28 stimulation (7, 8).

Besides CD28, CD80 and CD86 are also able to bind to another related receptor on T cells, CTLA4, with a higher affinity than for CD28 (9–11). Recent evidence has indicated that the role of CD152 (CTLA4) may be to deliver a negative signal (12) to an activated cell, resulting in anergy or cell death (13), which may be important in clearing cells from the site of an immune response (14). Further, mice deficient of CTLA4 die of massive T cell proliferation within a few weeks of birth (15, 16). Therefore, the relative expression of CD28 or CTLA4 on the surface of a T cell may determine whether the cell will become activated and continue to divide, become tolerant, or die.

The control of T cell homeostasis is complex and not completely understood. The lifespan of normal T cells is heterogeneous, as T cells have been shown to consist of multiple subsets of cells composed of both long-lived and short-lived cells (17). In humans receiving radiotherapy for nonlymphoid malignancies, the intermitotic survival time of naïve T cells is estimated to be about 3.5 years and for memory T cells, about 22 wk (18). To begin to gain an understanding of post-thymic T cell survival, it is necessary to know the replicative capacity of T cells as well as the intermitotic survival time. Because of the ability of CD28 to induce the cell survival gene Bcl-X_l (5), we began studies directed at developing a culture system that would allow the polyclonal proliferation of purified CD4$^+$ T cells. We thus developed a method to stimulate CD4$^+$ T cells that may provide for more optimal CD28 stimulation, with less "feeder cells" expressing B7-1 or B7-2 that might trigger CTLA4. Signal 1 is provided by a mAb directed against CD3, and the costimulatory signal is provided by a mAb directed against CD28 that does not cross-react with CTLA4. In addition, this system allows for long-term polyclonal expansion of HIV-infected CD4 cells (19) due to down-regulation of HIV coreceptors (7). We report that mixtures of adult naïve and memory CD4 cells can undergo about 30 to 40 population doublings (PDs).[3] The cells

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[2] Address correspondence and reprint requests to Dr. Bruce L. Levine, Mail Stop 061, Naval Medical Research Institute, 8901 Wisconsin Ave., Bethesda, MD 20889-5607.

[3] Abbreviations used in this paper: PD, population doubling; PDL, population-doubling level.

0022-1767/97/$02.00

retain a highly diverse TCR repertoire and can be induced to secrete mixtures of cytokines characteristic of Th1 or Th2 cells, depending on the form of CD28 stimulation. This system may be useful in adoptive immunotherapy and gene therapy protocols, not only for viral infections, but also for the treatment of malignancies in which the rapid expansion of purified T cells is desirable.

Materials and Methods

Antibodies

For cell purifications, the following purified and azide-free mAbs were used: anti-CD8 OKT8 (IgG2a), anti-CD11b OKM1 (IgG2b), anti-CD14 63D3 (IgG1), anti-CD16 3G8 (IgG1), anti-CD20 1F5 (IgG2a), and anti-HLA-DR 2.06 (IgG1). All of the hybridomas were obtained from the American Type Culture Collection (Rockville, MD) except 3G8, which was a kind gift of Dr. Stephen Shaw (National Institutes of Health, Bethesda, MD). For stimulations, anti-CD3 OKT3 (IgG1) and anti-CD28 9.3 (IgG2a) were used.

Cells

PBLs were isolated by Percoll gradient centrifugation from leukopacks obtained by apheresis of healthy donors. $CD28^+CD4^+$ T cells were purified as described previously (20) by a negative selection method using magnetic beads (Dynal, Lake Success, NY). In each experiment the purity of the separation was monitored by flow cytometry: $CD28^+$ $CD4^+$ T cells were >98% $CD3^+$, >98% $CD28^+$, and <3% $CD8^+$.

Long-term cell cultures

Cells were cultured in RPMI 1640 (BioWhittaker, Walkersville, MD) supplemented with 10% FBS (HyClone, Logan, UT), 2 mM L-glutamine (BioWhittaker), and 20 mM HEPES (BioWhittaker). OKT3 and 9.3 were bound to magnetic beads (Tosylactivated M-450; Dynal) at ~150 fg per bead (*cis* stimulation). In some experiments, as noted in figure legends, *trans* stimulation was conducted as follows: 1) Dynal beads were coated with anti-CD28 mAb 9.3 or anti-CD3 mAb OKT3, and equal mixtures of anti-CD3 and anti-CD28 beads added to cells; 2) for plastic-immobilized anti-CD3 stimulation, OKT3 was precoated on the culture wells or flask by overnight incubation with a 10 μg/ml solution. The culture wells or flasks were washed extensively with PBS before use and anti-CD28 stimulation performed by addition of 9.3 mAb at 1 μg/ml. Dynal beads were added to T cells at one to three beads per cell with the cells at 1×10^6 cells per/ml in complete medium. The cultures were fed at 2- to 3-day intervals to maintain a concentration of 1 to 2×10^6 cells/ml. Beads were not removed from culture, but were diluted progressively until restimulation. The cell cultures were counted and monitored for cell size or volume on a Coulter Counter model ZM and Channelyzer model 256 (Coulter, Hialeah, FL) equipped with a 70-μ long-bore orifice tube and restimulated with additional anti-CD3/anti-CD28-coated beads when the volume of the T cell blasts decreased to <400 femtoliters. For unstimulated cells, a lower gate was set at 25.5 femtoliters and for activated cells, a lower gate was set at 76.5 femtoliters so that paramagnetic beads would not be counted along with cells. Cell counts were determined from the total particles above these gates, and viability as assayed by trypan blue dye exclusion was routinely >95%. No exogenous cytokines or feeder cells were added to the cultures. Recombinant human IL-2 was added to certain cultures as indicated, but only after the cells did not respond to the addition of fresh anti-CD3/anti-CD28-coated beads by an increase in cell volume several days after the initial stimulation. PD time was determined by analysis of the exponential phase of cell growth by linear regression using statistical functions in SigmaPlot 3.0 and the formula PD in h = 24 × Log2/B1 where B1 = slope of the line number of cells plotted against days.

CDR3 size analysis to determine TCR Vβ diversity

RNA was extracted from 1×10^7 purified $CD4^+$ cells using TRIZOL Reagent (Life Technologies, Grand Island, NY) according to the manufacturer's instructions. cDNA was synthesized with the SuperScript Preamplification System for First Strand cDNA Synthesis kit (Life Technologies) according to the manufacturer's instructions using 5 μg of total RNA, oligo(dT) 12-18 (0.5 μg/μl), and Superscript II reverse transcriptase. The reaction was incubated at 42°C for 5 min, reverse transcriptase added with an additional 50-min incubation, and the reaction terminated at 70°C for 15 min. Residual RNA was removed by incubation with RNaseH at 37°C for 20 min.

CDR3 length of the variable β region was determined using PCR amplification originally described by Pannetier (21–23). cDNA from purified $CD4^+$ cells was amplified using Vβ region primers for each of 20 Vβ families and a Cβ region (24). Each PCR consisted of 1× Promega (Madison, WI) 10× buffer, 0.2 mM dNTP, 1.7 mM $MgCl_2$, 0.6 μM Cβ primer, 18 μg cDNA, and 0.5 U *Taq* coupled with TaqStart Ab (Clontech, Palo Alto, CA). The reaction was conducted in a 9600 Perkin-Elmer thermocycler (Norwalk, CT) for 40 cycles: 90°C for 25 s followed by 40 cycles of 90°C for 25 s, 60°C for 45 s, 72°C for 45 s, followed by an extension period at 72°C for 5 min. A 2-μl aliquot of this reaction was then submitted to a one-cycle runoff reaction using a 6-FAM-labeled Cβ primer. The samples were then mixed with deionized formamide, Rox-500 size standard (Perkin-Elmer), and dextran blue and subjected to electrophoresis on a 373 Applied Biosystem Sequencer (Foster City, CA) using a 24-cm well to read plates and an 8 M urea, 6% polyacrylamide gel. Data were then analyzed using the GeneScan Software 672 Analysis Software (Perkin-Elmer).

Cytokine assays

Cytokine contents in long-term cultures were determined upon restimulation by washing the cells in fresh medium, restimulation with beads, and collection of supernatants after 24 h. Concentrations of cytokines in cell-free supernatants were assayed by ELISA using commercially available kits obtained from the following sources: IL-2, T Cell Diagnostics/Endogen (Woburn, MA); IL-4, R&D Systems (Minneapolis, MN) or Dianova (Hamburg, Germany); IL-5, TNF-α, granulocyte macrophage-CSF, MIP-1α, MIP-1β, RANTES, R&D Systems; IL-10, Dianova; IL-13, Biosource (Camarillo, CA); IFN-γ, Endogen (Woburn, MA) or R&D Systems. All values reported were assessed by using dilutions of culture supernatant that yielded read-outs within the linear portion of the standard curve.

Results

CD28 costimulation specifically augments long-term $CD4^+$ T cell proliferation

Previous studies have shown that CD28 costimulation can specifically augment the proliferation of T cells during short-term culture. To more fully characterize the proliferative potential of adult $CD4^+$ T cells, cells were initially incubated with either anti-CD3 + anti-CD28 coimmobilized on beads, PHA + recombinant human IL-2 or anti-CD3 immobilized on beads plus recombinant human IL-2, as shown in Figure 1*A*. In earlier experiments, the ratio of anti-CD3 (OKT3) to anti-CD28 (9.3) conjugated to the beads was titrated and optimal long-term proliferation was observed at a ratio of 1:1 (data not shown). Only cells stimulated with the combination of anti-CD3 and anti-CD28 exhibited long-term growth. Cells cultured with optimal amounts of anti-CD3 plus recombinant human IL-2 or PHA plus recombinant human IL-2 exhibited similar growth rates for the initial 2 to 3 wk of culture compared with anti-CD3 plus anti-CD28-stimulated cells. However, the CD28-costimulated cells remained in exponential proliferation and the anti-CD3 plus recombinant human IL-2 and PHA plus recombinant human IL-2-stimulated cultures entered the plateau phase of growth in the 2nd to 3rd weeks of culture. Cell numbers in the experiment shown in Figure 1*A* had increased ~1 $\log_{10}$ more in cultures stimulated with anti-CD3 plus anti-CD28 than cultures stimulated with anti-CD3 + recombinant human IL-2 or PHA + recombinant human IL-2 by day 20 of culture. The input cells were typically >97% $CD28^+$, >95% $CD4^+$ (data not shown). It is important to note that APC were removed by negative immunomagnetic selection before the initiation of the culture. This was assured by the failure of the $CD4^+$ cells to grow in the presence of PHA alone. Furthermore, the proliferation induced by anti-CD3/CD28-coated beads was entirely autocrine, as exogenous cytokines or feeder cells were not added to the culture. Finally, the ability of anti-CD28 to sustain cell proliferation was specific, as beads coated with anti-CD3 and a variety of other Abs to T cell surface structures such as MHC class I, CD4, CD5, CD7, CD43, CD45, CD40L, and CTLA4 did not exhibit sustained proliferation (25, and data not shown). Thus, increased cell adhesion to the beads cannot account for specific enhancement of growth by CD28.

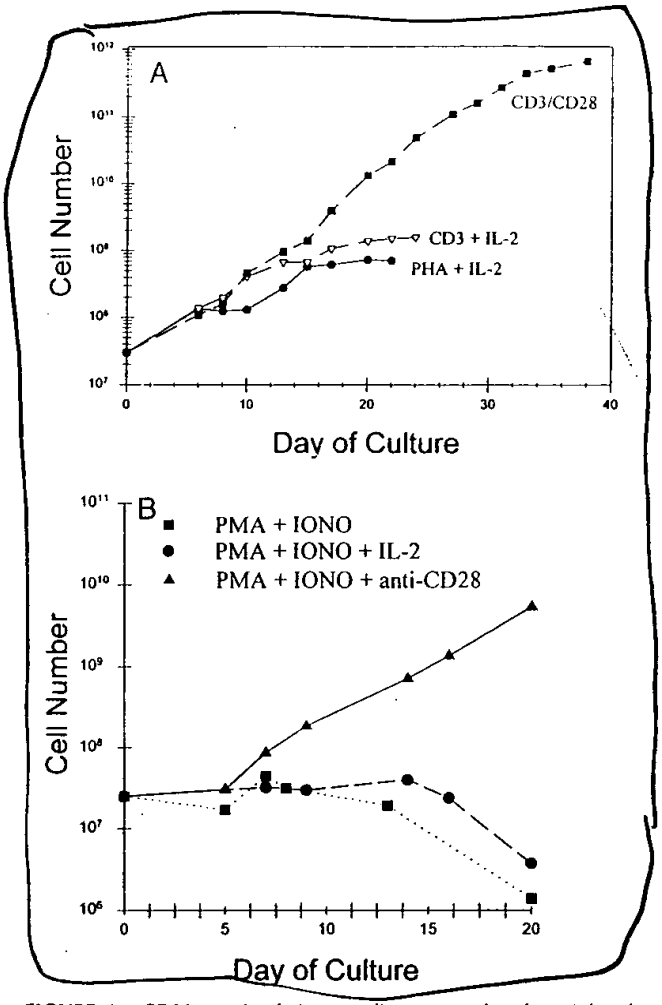

FIGURE 1. CD28 costimulation mediates growth of peripheral blood $CD4^+CD28^+$ T cells in the absence of exogenous cytokines or feeder cells and does not require Ag receptor stimulation. *A*, Anti-CD3 (OKT3) + anti-CD28 (9.3)-coated Dynal beads (solid squares), or anti-CD3 (OKT3)-coated Dynal beads + recombinant human IL-2 100 U/ml (open triangles), or PHA 5 μg/ml + recombinant human IL-2 100 U/ml (solid circles) were added to $CD4^+$ T cells. *B*, PMA 1.9 nM + ionomycin 0.08 μg/ml (solid squares), or PMA 1.9 nM + ionomycin 0.08 μg/ml + recombinant human IL-2 100 U/ml (solid circles), or PMA 1.9 nM + ionomycin 0.08 μg/ml + anti-CD28 (9.3)-coated Dynal beads (solid triangles) were added to $CD4^+$ T cells. Fresh medium was added to the cultures as required and excess cells discarded as described in *Materials and Methods*. Where indicated, recombinant human IL-2 was added to media for cells grown in recombinant human IL-2 to maintain a concentration of 100 U/ml. Cell number was determined using the average of two counts on a Coulter Counter ZM. The total number of cells that would be expected to accumulate is displayed, taking into account discarded cells.

To further delineate the specificity of anti-CD28 to induce long-term autocrine proliferation, $CD4^+$ T cells were stimulated with either PMA plus ionomycin, PMA plus ionomycin plus recombinant human IL-2, or PMA plus ionomycin plus anti-CD28 as shown in Figure 1*B*. Earlier experiments established optimal concentrations of PMA and ionomycin for T cell proliferation (data not shown). The addition of anti-CD28 prolonged and enhanced proliferation observed with PMA + ionomycin. Recombinant human IL-2 could not replace the effect of CD28 addition, similar to the result observed in Figure 1*A* in the context of anti-CD3 stimulation. The inability of recombinant human IL-2 addition to completely replace the growth-promoting effects of CD28 costimulation is likely related to the ability of CD28 and not IL-2 to induce sustained expression of the antiapoptotic gene Bcl-X_l (5). Another possibility is that CD28 may preserve the responsiveness of cells to IL-2 receptor stimulation. Recently, mouse T cell clones first stimulated with Ag and later by cross-linking the TCR were shown to become unresponsive to IL-2 (26). In addition, this experiment indicates that surface engagement of the TCR by anti-CD3 is not required for the growth-potentiating effects of CD28 costimulation, as pharmacologic "bypass" activation appears to be sufficient. Furthermore, a prolonged lag phase was observed in cultures of PMA plus ionomycin plus anti-CD28 stimulation, shown in Figure 1*B*, as revealed by comparing the lag phase in cultures stimulated with anti-CD3 and anti-CD28, shown in Figure 1*A*. The explanation for the increased lag phase in PMA/ionomycin-stimulated cultures is not fully known, but is likely related to the observation that only a subset of cells bearing CD101 responds to anti-CD28 and PMA stimulation, while essentially all $CD28^+$ cells respond when stimulated with anti-CD3 plus anti-CD28 (27). Together, these experiments confirm and extend previous results showing that CD28 delivers a signal that costimulates T cells stimulated by anti-CD3 or by phorbol esters plus calcium ionophore and results in autocrine proliferation for about 1 mo (28).

Cyclical restimulation of $CD4^+$ T cells with immobilized anti-CD3/CD28 results in long-term exponential growth

In the absence of exogenous recombinant human IL-2 addition we have previously shown that $CD4^+$ T cells stimulated with anti-CD3/CD28 will exhibit exponential growth for about 20 days (28), while $CD4^+CD45RA^+$ (naïve) T cells will exhibit exponential growth for about 45 days (29). In addition, we found that repeated addition of beads coated with anti-CD3/CD28 was necessary to sustain cell proliferation, and that addition of exogenous recombinant human IL-2 could further sustain proliferation. In Figure 2*A*, the growth curve of $CD4^+$ T cells cyclically stimulated by anti-CD3/CD28 beads, as described in *Materials and Methods*, is shown. After the fifth restimulation, the cells are no longer able to maintain logarithmic growth and plateau at 8 $\log_{10}$ above the input number of cells, corresponding to a mean population-doubling level (PDL) of 27. In contrast, the period of exponential growth could be extended to >60 days when recombinant human IL-2 was added to the culture. In the experiment shown in Figure 2*A*, the exponential growth phase was extended to a 2×10^{10}-fold increase, or a PDL of 34, with the addition of exogenous recombinant human IL-2 on day 49 of culture, at a point when the cells had become unresponsive to repeated CD3/CD28 stimulation alone. The phenotype of the cells remained >99% TCR-$\alpha\beta^+CD4^+$ (data not shown). The diversity of the TCR expressed in the cells is shown below.

Summarized in Table I are durations of culture and fold expansion of 10 independent $CD4^+$ T cell cultures performed as described in *Materials and Methods*. The cells could be periodically restimulated with anti-CD3/CD28 beads with or without the addition of exogenous recombinant human IL-2, which was added when the culture was unresponsive to further restimulation with anti-CD3/CD28 alone. In the absence of recombinant human IL-2 addition, the cells had an average 2×10^5-fold expansion and an average duration of culture of 39 days. Long-term CD4 proliferation could be obtained with the combination of CD3/CD28 and recombinant human IL-2 stimulation of CD4 cells, with an average duration of proliferation of 82 days. The proliferation was exponential in all cultures as indicated by the semilog plot of cell number vs days of culture. In the cultures grown in the absence of exogenous recombinant human IL-2, the average PD time was

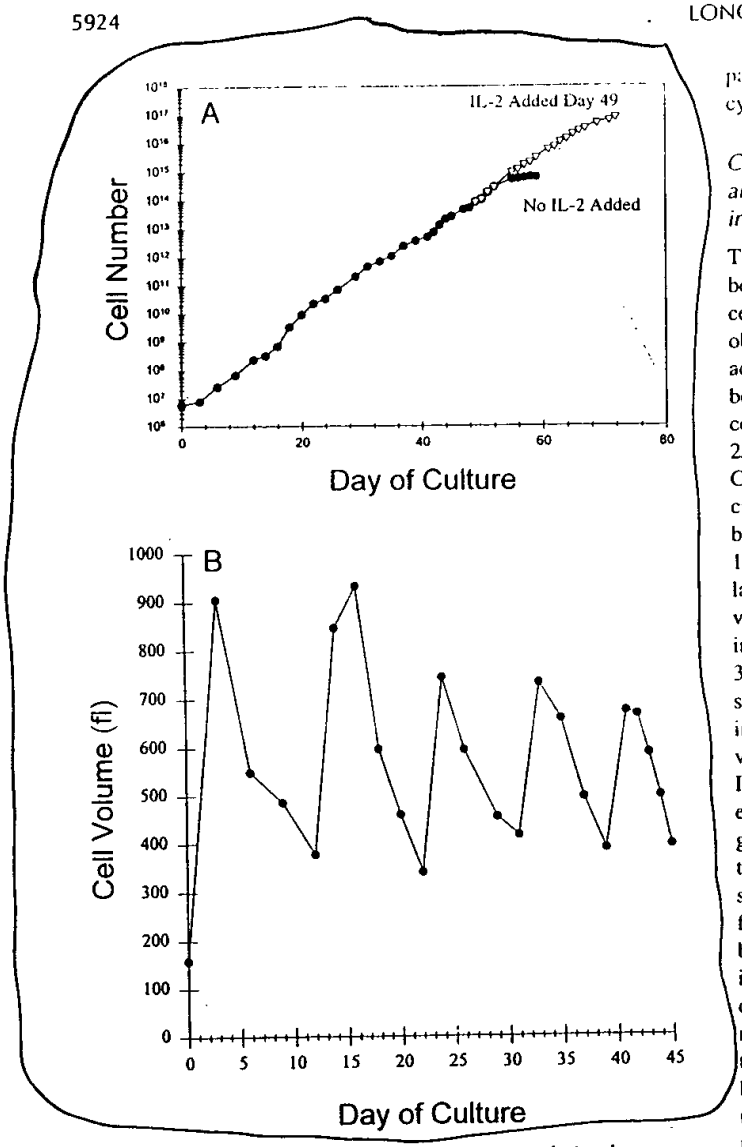

FIGURE 2. Cyclic changes in cell volume occur during long-term exponential proliferation of CD4$^+$ T cells mediated by stimulation with anti-CD3 plus anti-CD28 immobilized on beads. *A*, Growth of CD4$^+$ T cells following six cycles of bead restimulation without the addition of exogenous cytokines (closed circles); the same experiment with the exception of the addition of exogenous recombinant human IL-2 on day 49 (closed circles followed by open triangles). *B*, Mean cell volume (femtoliters) of CD4$^+$ T cells cyclically restimulated with anti-CD3 + anti-CD28-coated beads. At the initiation of the culture, peripheral blood CD4$^+$ T cells had a mean cell volume of 159 femtoliters. Cells were restimulated on days 12, 22, 31, 39, and 45 when the mean cell volume fell below 400 femtoliters. Volume was measured on a Coulter Channelyzer Model C256. For resting day 0 cells, a gate was set to exclude particles <65 femtoliters; for activated cells, a gate was set to exclude particles <200 femtoliters to ensure that beads in the culture were not sized.

37.9 h. In contrast, during the IL-2-dependent phase of cell expansion, the PD time was somewhat slower at 53.2 h. The average PDL for adult CD4$^+$ T cell cultures with exogenous recombinant human IL-2 added was 33 (1×10^{10}-fold expansion). The highest PDL obtained following anti-CD3/CD28 stimulation + recombinant human IL-2 of CD4$^+$ naïve T cells was 41.6 (data not shown). Thus, CD3/CD28 stimulation allows for substantial autocrine-driven proliferation followed by a period of proliferation that is paracrine and dependent upon the addition of exogenous cytokines.

Cyclical restimulation of CD4$^+$ T cells with immobilized anti-CD3/CD28 results in cyclical changes in cell volume independent of proliferative rate

The above results established that exponential proliferation could be maintained for many weeks by periodic restimulation of CD4 cells with anti-CD3/CD28-coated beads. As mentioned earlier, we observed that prolonged exponential proliferation could be achieved if cells were periodically restimulated with Ab-coated beads when cells approached resting cell volumes. In Figure 2*B* the cell volumes are displayed for the growth curve shown in Figure 2*A*. Resting T cells have a mean cell volume of ~170 femtoliters. CD4$^+$ T cells stimulated with immobilized anti-CD3/CD28 increased in volume from 170 femtoliters to nearly 900 femtoliters by 3 days. The cell volume gradually declined over the course of 12 days to 377 femtoliters. At this point, the cells were restimulated by the addition of fresh anti-CD3/CD28 beads and the cell volume again returned to near 900 femtoliters. The culture shown in Figure 2 was restimulated five times, on culture days 12, 22, 31, 39, and 45 before these cells became unresponsive to further restimulation by the addition of anti-CD3/CD28 beads. By comparing Figure 2, *A* and *B*, it is apparent that cyclic changes in cell volume occur that are independent of the rate of cell proliferation. It has been commonly thought that T cell clones need to be "rested" before restimulation. Our results indicate that plateaus in the growth curve are not necessary for cells to regain responsiveness to restimulation. However, a decrease in cell volume appears to serve as an indication that the cells have become responsive to further restimulation. Using this cell stimulation protocol, we have been unable to detect a change in the growth rate of the cells that is related to the readdition of Ab-coated beads. Thus we have concluded that CD4 cell proliferation kinetics can remain exponential and independent of the periodic changes in cell volume. A trivial explanation for the periodic changes in cell volume would be that the beads were included in the cell sizing process, as particles bound to the cell. The beads have a volume that is similar to that of resting cells, and the beads can be electronically gated out from analysis of activated, but not resting, cells. The following observation indicates that the addition of beads does not account for the periodic changes in cell volume, as the beads could be removed from the cell culture on day 3 of culture by magnetic separation, and the beadfree cells were still found to exhibit the cell enlargement and gradual return to near resting volume. Finally, if cells were allowed to grow in culture until their size returned to resting cell volumes, they became unresponsive to restimulation, and cell loss from apoptosis began to occur (data not shown).

Stimulation with immobilized anti-CD3/CD28 of CD4$^+$ T cells results in polyclonal proliferation

To determine whether the cell growth induced by immobilized anti-CD3/CD28 was polyclonal, 10 mAbs directed at 7 different TCR Vβ families covering about 40% of the Vβ repertoire were used to stain cells at the beginning and at day 71 of the culture. As shown in Figure 3, anti-CD3/CD28 stimulation was able to maintain a polyclonal population of CD4$^+$ T cells for 71 days, as all tested Vβ families remained present and there was no variation by more than twofold in the abundance of any given family. The CD4$^+$ T cells shown in Figure 3 are from the same culture shown in Figure 2*B*, so that after 71 days these cells had a mean PDL of 33.2.

Table I. *Summary of proliferative rates of adult CD4⁺ T cells stimulated by immobilized anti-CD3 plus anti-CD28 for 10 separate experiments*

Exp.	Medium	Duration of Culture (days)	Fold Expansion	Population Doubling Time (hours)	Exponential Growth (days)	Correlation Coefficient
1	No IL-2	33	1.8 E + 04	44	14	0.991
2	No IL-2	34	3.2 E + 05	35	17	0.980
3	No IL-2	40	8.3 E + 04	42	19	0.996
4	No IL-2	44	2.3 E + 05	35	16	0.988
5	No IL-2	44	5.0 E + 05	33	16	0.982
Average		39	2.3 E + 05	38	16	0.987
6	IL-2 Added Day 33	65	8.5 E + 05	59	27	0.995
7	IL-2 Added Day 28	76	5.0 E + 09	41	45	0.994
8	IL-2 Added Day 49	78	4.6 E + 10	48	61	0.996
9	IL-2 Added Day 31	84	4.4 E + 08	64	69	0.997
10	IL-2 Added Day 41	106	4.1 E + 08	54	33	0.993
Average		82	1.0 E + 10	53	47	0.995

Cells were stimulated in the absence of feeder cells as indicated in *Materials and Methods*, and for experiments 1–5 cells were grown for the duration of the culture without the addition of exogenous cytokines. For experiments 6–10 cells were grown without the addition of exogneous cytokines until recombinant human IL-2 was added on the indicated day, as exemplified in Figure 2*A*. The proliferative capacity was not assessed in experiments 1–5 as the cultures were terminated while cells remained in exponential growth, whereas experiments 6–10 were terminated when cell cultures began to display a plateau phase. Population doubling (PD) time was determined by analysis of the exponential phase of cell growth by linear regression as described in *Materials and Methods*. The cultures had exponential growth as indicated by the goodness of fit of the growth curve with a straight line. The duration of exponential proliferation is indicated.

To further analyze the effect of prolonged CD4 cell culture on the TCR repertoire, a PCR approach based upon measurement of the length of CDR3 was used. CD4 cells were cultured for 60 days in the presence of anti-CD3 and CD28-coated beads and the TCR clonotypes of 20 Vβ families determined by RNA PCR analysis (22, 30). The PCR products were analyzed on an automated DNA sequencer and the intensity of the bands depicted on plots with the heights corresponding to the intensity of the fluorescent bands. The Vβ repertoire as assessed at the start of culture was diverse as each Vβ family had a gaussian distribution pattern of an average of eight peaks separated by three nucleotides (Fig. 4). Visual inspection after 60 days of culture indicated that a less heterogeneous profile of CDR3 sizes was present. About 75% of the Vβ families remained with diverse profiles while the remaining showed clonal peaks that suggested oligoclonal expansions. Thus, prolonged culture of CD4 cells with anti-CD3 and CD28 can result in the clonal contraction or expansion that is not apparent with FACS analysis. However, this high resolution analysis indicates that the culture retains much of the input diversity.

Cytokine mRNA and protein is more abundant in CD4⁺ T cells stimulated with anti CD3 + anti-CD28 than with anti-CD3 + recombinant human IL-2

It has previously been shown that CD28 stimulation results in increased cytokine production through stabilization of cytokine mRNA as well as through increased de novo synthesis (31). Figure 5 shows that the induction of IL-2 and TNF-α mRNA following anti-CD3/CD28 stimulation is much more pronounced and prolonged than mRNA induced following anti-CD3 + recombinant human IL-2 stimulation. The enhanced cytokine expression did not reflect differences in growth rate, as the cell proliferation was equivalent for the first several weeks of culture, as shown in Figure 1. Cytokine gene expression was not constitutive, as mRNA for IL-2 and TNF was not detected on day 8 before restimulation. However, inducible cytokine mRNA expression was observed on restimulation. To determine whether this increase in cytokine mRNA correlated with secretion, supernatants from CD4⁺ T cells stimulated with anti-CD3 beads + recombinant human IL-2 or anti-CD3/CD28 beads were collected after 24 h and assayed for a variety of cytokines and chemokines by ELISA, as shown in Figure 6. Anti-CD3/CD28 not only induced higher levels of most cytokines by significant amounts, but also induced the secretion of detectable levels of IL-4 and IL-13. The pattern of lymphokine production reflected what has been seen in Th0/Th1 cells (32, 33). Shown in Table II is the level of cytokine contained in supernatants from anti-CD3/CD28-stimulated cells from the CD4 cells shown in Figures 2 and 3. Supernatants were collected 24 h after the first four stimulations with anti-CD3/CD28 beads and corresponding to the day of culture shown in the table. Levels of IL-2 and IFN-γ induced remained high compared with IL-4, indicating the maintenance of a Th0/Th1 phenotype. Earlier studies have shown that long-term culture of T cells with anti-CD3 and anti-CD28 can lead to a population of cells that secretes predominantly Th2-type cytokines (34). However, these studies were performed with anti-CD3 and anti-CD28 in *trans*, that is, with anti-CD3 immobilized on plastic and soluble anti-CD28. If anti-CD3 and anti-CD28 are presented in *cis* (immobilized on the same bead), the present results suggest that a progressive bias toward Th2 cytokine patterns does not occur. These results suggest that the manner in which CD3 and CD28 are stimulated on the surface of T cells results in differential signal strengths or in differential signals generated through the TCR and CD28 that can elicit distinct patterns of cytokine secretion. These findings may prove useful in adoptive immunotherapy approaches where skewing toward a particular cytokine profile is desirable.

Discussion

Conditions have been developed that permit extensive in vitro CD4 cell propagation. Although we are not aware of previous studies documenting long-term (>4 wk) proliferation of polyclonal CD4 cells, anti-CD3 plus anti-CD28 mAbs have been used by others to clone T cells with high efficiency. Riddell and Greenberg (35) cloned CMV-specific CD8⁺ T cells and maintained these cells for 3 mo in culture, at which time they maintained their Ag-lytic activity. Another study has demonstrated cloning of CD4⁺CD45RO⁻ T cells under feeder cellfree conditions. However, the addition of exogenous cytokines was required (36). CD2 stimulation has also been utilized in concert with CD28 stimulation to induce long-term autocrine proliferation of CD4⁺ T cell clones (37).

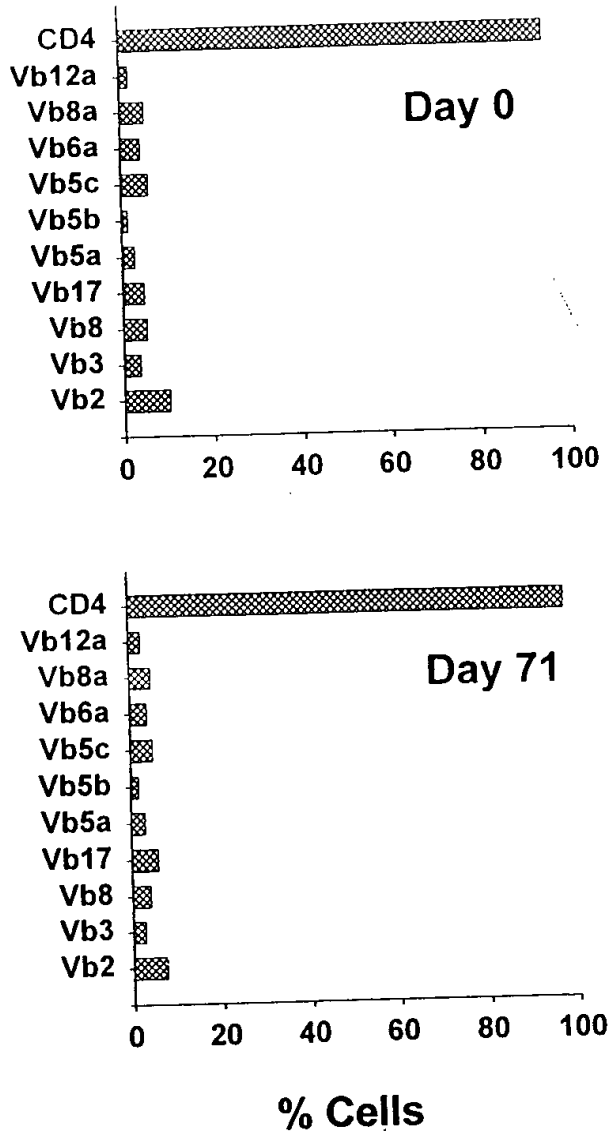

FIGURE 3. Assessment of TCR Vβ repertoire by FACS after anti-CD3- and anti-CD28-mediated proliferation. Polyclonality was assessed by FACS analysis using mAbs to TCR Vβ families with 10 different mAbs (T Cell Diagnostics and Immunotech) covering 7 different TCR Vβ families as well as an anti-CD4 mAb. Cells from the experiment shown in Figure 2 were stained on the day of initiation of the culture (day 0) and after 71 days in culture. Day 71 of the culture corresponds to a 10 $\log_{10}$-fold expansion or 33.2 mean PDs. The percent positive cells was designated as those with fluorescence intensities greater than 98% of cells stained with phycoerythrin- or FITC-conjugated isotype-specific normal mouse Ig as control. Analysis was performed on a Coulter Epics Elite.

Long-term growth of $CD4^+$ T cells following CD3/CD28 stimulation followed a predictable pattern. Exponential growth was observed throughout the culture. In spite of this the cells exhibited periodic changes in cell volume. The basis for the cyclic changes in cell size remain unknown but may relate to differences of cell cycle distribution. Alternatively, the changes in cell size may indicate a previously unrecognized cell growth function that is regulated by CD3/CD28 stimulation. In support of this, we have noted that cells stimulated in costimulation-deficient conditions, such as with mitogenic lectins and recombinant human IL-2, proliferate at equivalent rates and maintain smaller cell volumes. Cell sizing is useful for determining when to restimulate the cultures, thereby avoiding the plateau in growth curves that occurs with standard culture techniques. This is one factor that is important in avoiding apoptosis and maintaining TCR repertoire during prolonged in vitro culture.

Early in the cultures, cells secreted large amounts of IL-2 and other cytokines, allowing for autocrine proliferation in the absence of added cytokines. However, cells eventually required the addition of exogenous recombinant human IL-2 to continue proliferating. Whether this progression from an autocrine to a paracrine state is a natural state of differentiation of peripheral blood-derived $CD4^+$ T cells or a reflection of the in vitro aging induced by this method of culture is currently under investigation. We have observed that CD28 receptor levels decline when $CD4^+$ T cells were cultured for several weeks (data not shown). The absence of CD28 on the cell surface would mean that a costimulatory signal could not be delivered by anti-CD3/CD28-coated beads and thus would account for the necessity of adding exogenous recombinant human IL-2. In fact, the decline in CD28 expression with age (38) and during progression to AIDS (39–42) is well documented. Following the induction of anergy in either resting or activated T cells, CD28 is down-regulated at the level of mRNA (43). Recently Lloyd et al. (44) showed that IL-4 could down-modulate CD28 expression on $CD8^+$ T cells. These $CD28^-$ cells were found to be less responsive to anti-CD3-mediated proliferation than $CD28^+CD8^+$ T cells.

Immobilizing anti-CD3 and anti-CD28 on beads allowed the titration of the signal delivered through the TCR/CD3 complex with the signal delivered through the CD28 receptor. We found that soluble anti-CD28 did not support long-term proliferation equivalent to immobilized anti-CD28. Preliminary studies suggest that this is due to the ability of immobilized anti-CD28 to maintain CD28 expression, whereas soluble CD28 leads to a more rapid loss of CD28 expression (data not shown). The fact that earlier studies have used soluble anti-CD28 may be the primary reason that long-term polyclonal proliferation of CD4 cells has not previously been reported. Timing, or the delivery, of the signal to the TCR as well as accessory molecules can affect cellular responses such as proliferation and apoptosis (45, 46). For this reason we titrated the relative amounts of anti-CD3 (OKT3) and anti-CD28 (9.3) on beads and found that for long-term growth of $CD4^+$ T cells, a ratio of 1:1 was optimal (data not shown). Similarly we determined the optimal ratio of beads to cells for long-term growth and found that there was no difference between 3 beads per cell and 1 bead per cell, but at 0.3 beads per cell long-term growth was not sustained (data not shown). This signal was independent of CD3 ligation per se, as pharmacologic simulation of CD3 stimulation by phorbol ester and calcium ionophore was also enhanced by the addition of anti-CD28 mAb.

CD28 stimulation delivered a specific signal that enhanced cell growth compared with stimulation with anti-CD3 alone. The initial rates of proliferation were equivalent in CD3 and recombinant human IL-2-stimulated cultures compared with CD3 and CD28-stimulated cultures. However, cells stimulated with optimal amounts of anti-CD3 in the presence of exogenous recombinant human IL-2 did not maintain prolonged growth. These results are in agreement with previous studies in the mouse indicating that Ag-dependent clonal expansion of $CD4^+$ T cells in vivo is dependent on CD28 costimulation (47). This may be due to the ability of CD28 to promote lymphocyte survival in both human and mouse T cells (5, 6).

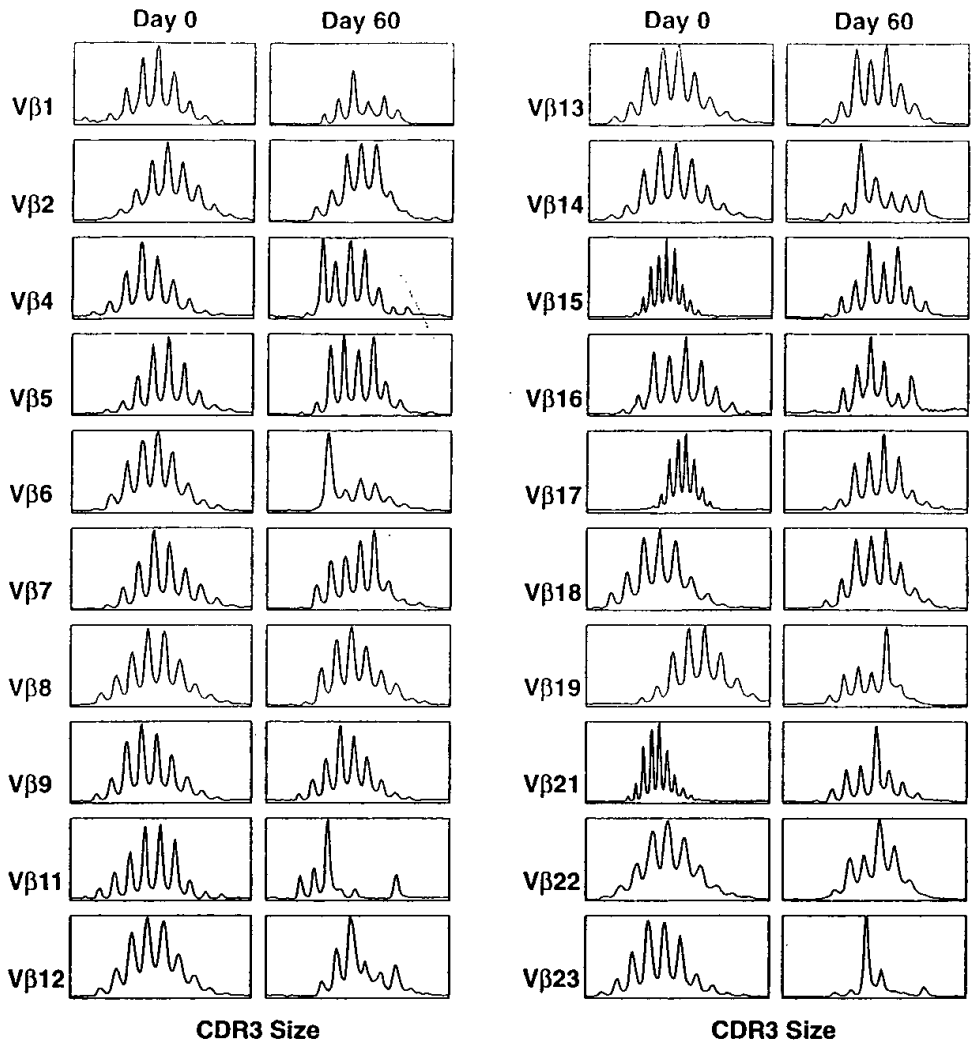

FIGURE 4. Assessment of TCR Vβ repertoire by PCR after anti-CD3- and anti-CD28-mediated proliferation. Polyclonality was assessed by CDR3 length of the variable β region for 20 Vβ families and a Cβ, as described in *Materials and Methods*. Resting $CD4^+$ T cells isolated from an apheresis product (day 0) and CD3/CD28-stimulated cells on day 60 of culture were analyzed. A gaussian distribution of the eight CDR3 length fragments indicates a polyclonal population, and a nongaussian distribution indicates either an expansion or deletions in particular Vβ subsets within the same family.

The ability to propagate Ag-nonspecific or polyclonal T cell lines first became possible following the identification and characterization of IL-2 as a T cell growth factor (48). However, mixed populations of $CD4^+$ and $CD8^+$ would eventually result in a population of cells that was predominantly $CD8^+$ (49). Furthermore, polyclonal T cell propagation has not been demonstrated with IL-2, possibly due to the ability of IL-2 to prime activated T cells for apoptosis. Subsequent studies of the long-term growth of human T cells have necessitated the use of IL-2 in addition to mitogenic lectins and autologous or irradiated allogeneic feeder cells (37, 50, 51).

Our present studies indicate that polyclonal populations of adult CD4 cells can proliferate for a 10^9- to 10^{11}-fold expansion, equivalent to 30 to 40 PDs. Previous studies by Effros and coworkers have addressed the lifespan of T lymphocytes in vitro (50). They reported a mean PD of 23 ± 7 from adult T cells using lectins and feeder cells for cell propagation. We have consistently achieved higher PDLs. Furthermore, while some reports indicate that rare T cells can grow in vitro for up to 80 PDs (52, 53), our present results indicate that this does not appear to reflect the replicative capacity of the vast majority of polyclonal peripheral T lymphocytes. Thus, our studies indicate that the replicative capacity of CD4 cells is extensive but finite. In separate studies we found heterogeneous replicative properties of CD4 cells as we found that naïve cells had a greater replicative capacity than memory CD4 cells (29). In vitro, costimulation by anti-CD28 appears to have a role in telomerase induction (54); however, it remains to be established whether this has any function in determining the long-term proliferative capacity of the cultures that we observe in vitro or in determining T cell replicative capacity in vivo.

The role of CD28 costimulation in T cell differentiation remains controversial. In mice, CD28 costimulation is required to prime Th1 and Th2 cells; however, CD28 appears to promote the differentiation of cells that secrete Th2 cytokines (55). We have examined cytokine production in cultures following stimulation with beads bound with both anti-CD3 and anti-CD28 (*cis* stimulation) and found a maintenance of a Th1 profile of cytokine production over several rounds of stimulation during

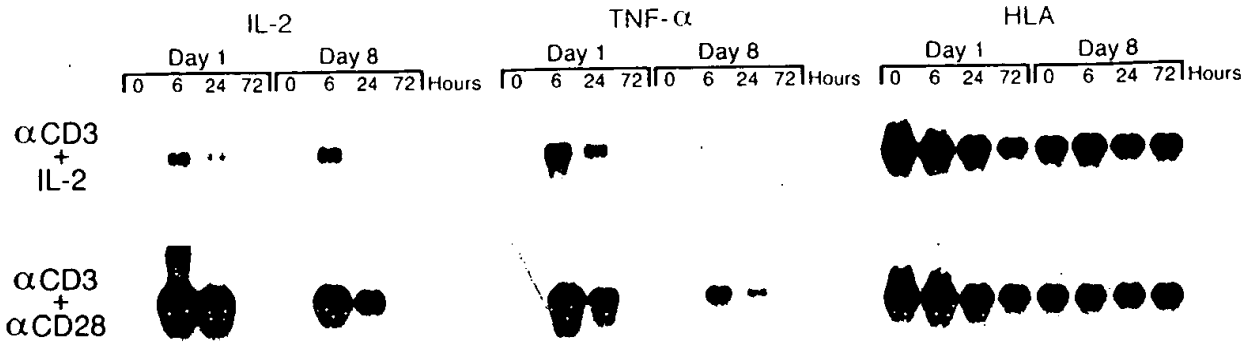

FIGURE 5. Cytokine gene expression remains inducible in cultures of exponentially proliferating $CD4^+$ T cells after anti-CD3 plus anti-CD28 stimulation. $CD4^+CD28^+$ T cells were stimulated and maintained in culture as described in *Materials and Methods*. On day 1 or 8 of the culture, cells were collected and subjected to Northern analysis at 0, 6, 24, and 72 h following stimulation with either anti-CD3 + recombinant human IL-2 100 U/ml or plastic-immobilized anti-CD3 plus anti CD28 1 μg/ml for IL-2, TNF-α, and HLA class I mRNA expression as described in *Materials and Methods*.

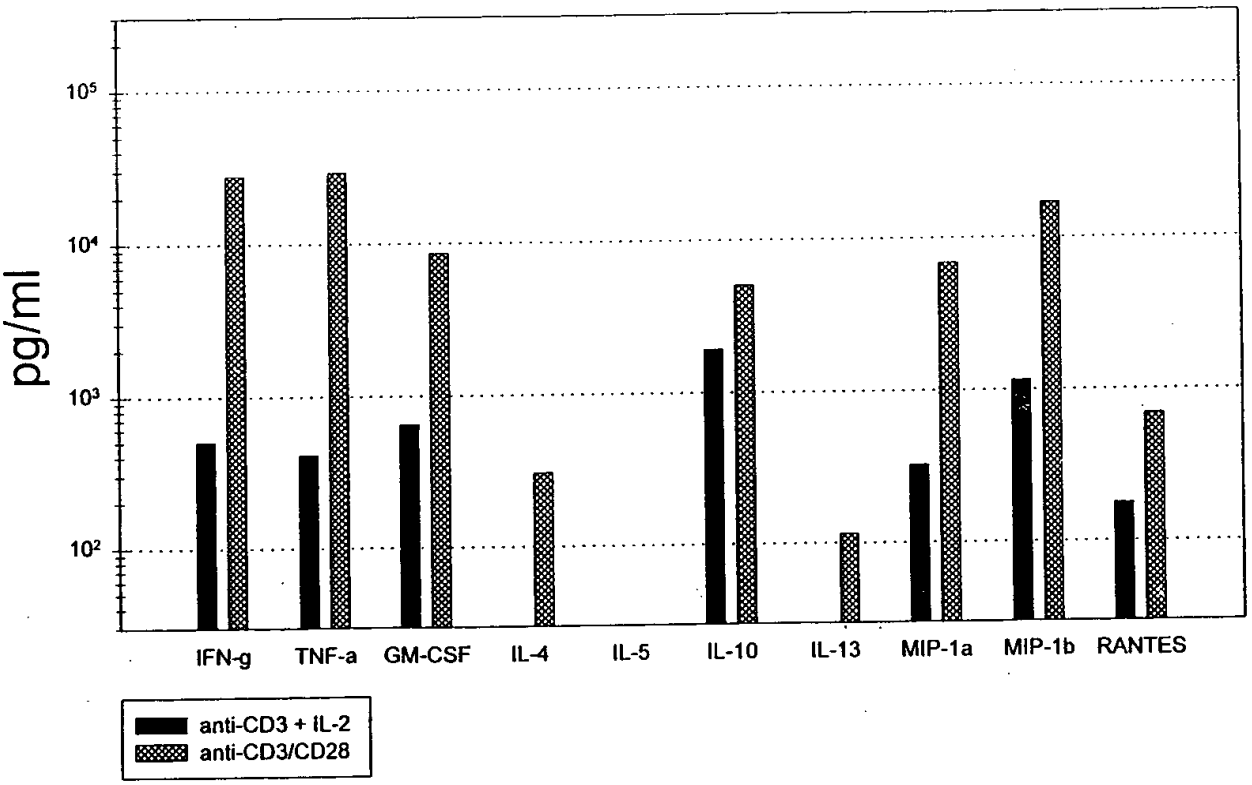

FIGURE 6. Cytokine and β chemokine secretion in cultures of $CD4^+$ cells as measured by ELISA. Anti-CD3- plus anti-CD28-coated beads or anti-CD3-coated beads plus recombinant human IL-2 100 U/ml were added to freshly isolated peripheral blood $CD4^+CD28^+$ T cells at a ratio of three beads per cell. IL-2 as measured by ELISA was 189,600 pg/ml for anti-CD3- plus anti-CD28-coated beads and 40,812 pg/ml for anti-CD3-coated beads plus recombinant human IL-2 100 U/ml. Supernatants for cytokine analysis were collected 24 h later and appropriate dilutions analyzed by ELISA as described in *Materials and Methods*. Sensitivity of the ELISA kits were as follows: IFN-γ, 15.6 pg/ml; TNF-α, 15.6 pg/ml; granulocyte macrophage-CSF, 15.6 pg/ml; IL-4, 15.6 pg/ml; IL-5, 15.6 pg/ml; IL-10, 15.6 pg/ml; IL-13, 19.5 pg/ml; MIP-1α, 31.25 pg/ml; MIP-1β, 31.25 pg/ml; and RANTES, 31.25 pg/ml.

long-term culture. This confirms previous studies that costimulation in *cis* is more efficient than in *trans* (56). Although the mechanism leading to these results remains unknown, we favor the notion that different strengths of Ag dose and costimulation can lead to distinct Th1 and Th2 differentiation. It is also possible that these different forms of stimulation lead to selective survival of Th1- or Th2-like cells, and further studies will be required to distinguish these possibilities. Together, the present results help clarify apparent differences from previous studies of cytokine secretion patterns after CD28 stimulation where repetitive *trans* stimulation could lead to the emergence of a Th2 phenotype (34), while repetitive *cis* stimulation maintained cytokine secretion patterns consistent with a Th1 phenotype (19, 28). Regardless of the mechanism involved, it is possible that these results might be useful to produce polyclonal populations of T cells that have Th1 or Th2 bias.

Table II. *Cytokine secretion from CD4⁺ T cells after four cycles of re-stimulation with immobilized anti-CD3 plus anti-CD28*

Stimulation	Day of Culture	IL-2	IFN-γ	IL-4
1	1	18,875	2,083	<62.5
2	13	40,682	17,917	173
3	23	19,277	14,229	267
4	32	10,099	6,993	<62.5

Supernatants were collected 24 h after each stimulation. For stimulations 2, 3, and 4, cells were washed out of conditioned media and reseeded into fresh media in order to measure de novo cytokine production. Values shown are in pg/ml. Sensitivity of the ELISA kits were as follows: IL-2, 61 pg/ml; IFN-γ, 40 pg/ml; IL-4, 62.5 pg/ml.

Many lines of evidence point to an impairment of the cellular immune system with increasing age. Engwerda, Handwerger, and Fox (57) have shown that the response of both CD4 and CD8 or naïve and memory cells to CD28 costimulation is impaired in aged mice. However, in aged humans there is a decline in CD28 expression (38), and a recent study has detected clonal expansions within certain TCR Vβ subsets in aged humans (58). One reason for decreased immune function or repertoire in the elderly is thought to be related to the decrease in thymic export of T cells following involution. In a study of CD4⁺ T cell regeneration following chemotherapy, Mackall et al. (59) found that age or thymic volume correlated with the ability and speed of CD4 cell counts to return to pretreatment levels. Thus, the ability of peripheral T cells to expand ex vivo and reconstitute an impaired immune system has remained in doubt. The data presented here provide further evidence that substantial ex vivo expansion of polyclonal CD4⁺ T cells is possible following cyclical stimulation with immobilized anti-CD3 and anti-CD28 mAbs. Highly diverse populations of CD4 cells could be maintained for at least 8 wk in cultures. All results are compatible with the notion that this approach leads to physiologic cell growth. Furthermore, in experiments involving cells from more than 100 donors, we have not observed transformation after CD3 and CD28 stimulation. Thus, this approach should permit adoptive immunotherapy and gene therapy strategies for immunodeficiencies and malignancies, as well as facilitate further studies on the replicative capacity of T cells.

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Antiviral Effect and Ex Vivo CD4$^+$ T Cell Proliferation in HIV-Positive Patients as a Result of CD28 Costimulation

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Because stimulation of CD4$^+$ lymphocytes leads to activation of human immunodeficiency virus–type 1 (HIV-1) replication, viral spread, and cell death, adoptive CD4$^+$ T cell therapy has not been possible. When antigen and CD28 receptors on cultured T cells were stimulated by monoclonal antibodies (mAbs) to CD3 and CD28 that had been immobilized, there was an increase in the number of polyclonal CD4$^+$ T cells from HIV-infected donors. Activated cells predominantly secreted cytokines associated with T helper cell type 1 function. The HIV-1 viral load declined in the absence of antiretroviral agents. Moreover, CD28 stimulation of CD4$^+$ T cells from uninfected donors rendered these cells highly resistant to HIV-1 infection. Immobilization of CD28 mAb was crucial to the development of HIV resistance, as cells stimulated with soluble CD28 mAb were highly susceptible to HIV infection. The CD28-mediated antiviral effect occurred early in the viral life cycle, before HIV-1 DNA integration. These data may facilitate immune reconstitution and gene therapy approaches in persons with HIV infection.

CD4$^+$ T cells contain the major reservoir of HIV-1 in vivo (*1*, *2*). The difficulties involved in inducing proliferation in vitro of autologous CD4$^+$ T cells from patients with HIV-1 infection limit the therapeutic potential for many approaches that involve gene therapy or immune reconstitution of infected persons (*3*). Two obstacles attributed to impaired CD4$^+$ T cell proliferation are a limited clonogenic potential of the uninfected CD4 and CD8 cells and the activation of HIV-1 expression and viral production (*4*). In addition to T cell receptor (TCR) engagement of an antigenic peptide bound to major histocompatibility complex (MHC) receptors, other costimulatory signals are necessary for T cell activation. The most important of the costimulatory signals identified to date is provided by the interaction of CD28 on T cells with its ligands CD80 and CD86 on antigen-presenting cells (*5*). Because CD28 signal transduction can prevent apoptosis in cultures of HIV-infected cells and can induce expression of the Bcl-X_L cell survival gene (*6*), we tested the hypothesis that costimulation might be limiting in cultures from HIV-infected patients.

We cultured lymphocytes from 10 patients with HIV-1 infection (CD4 counts of 350 to 600 cells/mm^3) in the presence of beads coated with CD3 mAb OKT3 and CD28 mAb 9.3 (Table 1). Cell culture was performed in the absence of exogenous cytokines or feeder cells, as CD28 stimulation provides the necessary costimulus to replace feeder cells (*7*). Figure 1 shows the growth curve of CD4$^+$ T cells from an HIV-infected patient after stimulation by a conventional method [with phytohemagglutinin (PHA) and interleukin-2 (IL-2)] or with CD3 and CD28 mAbs in medium that did not contain antiretroviral agents. In the PHA-stimulated culture, the growth curve revealed an initial exponential expansion and a subsequent plateau phase, resulting in termination on day 18 of the culture (Fig. 1A). This pattern was coincident with increased p24 antigen production and with increased viral burden as measured by a quantitative polymerase chain reaction (PCR) for cellular HIV-1 *gag* (Fig. 1, B to D). In contrast, when cells were cultured with CD3 and CD28 mAbs, exponential cell proliferation was maintained for 50 days (Fig. 1A). Although there was evidence of modest viral expression early in the culture, as indicated by the concentration of p24 on day 8 (Fig. 1B), viral production and proviral DNA decreased to undetectable amounts in the culture (Fig. 1, C and D). Similar results were obtained whether the starting cell population was peripheral blood mononuclear cells (PBMCs) or purified CD4$^+$ T cells; this finding suggested that the enhanced cell proliferation and antiviral effects in the culture stimulated with CD3 and CD28 mAbs were not dependent on CD8$^+$ T cells or accessory cells (Table 2) (*8*).

A quantitative PCR was used to determine amounts of HIV-1 *gag* DNA and RNA in the cultures of lymphocytes from the 10 HIV-positive patients (Table 1). Culture with CD28 mAb resulted in decreased viral burden in all patient-derived cells, including the cells cultured in the absence of antiretroviral agents. HIV-1 *gag* proviral DNA became undetectable in six of seven cultures from patients that were cultured in the absence of antiretroviral agents, and HIV-1 *gag* RNA became undetectable in five of the seven cultures. Culture supernatants were also sampled for p24 antigen at 7- to 14-day intervals. Antigen was not detected in 9 of the 10 patients; in one patient (patient 9; Table 1 and Fig. 1), decreasing concentrations of p24 antigen with time were detected. Virus-free CD4$^+$ T cell proliferation also occurred even when CD8 cells constituted <1% of the cells (Table 2) (*9*).

The increase in the number of CD4 cells was not significantly different in the presence or absence of a combination of antiretroviral agents in three patients (*8*). In uninfected adult blood donors, the average absolute magnitude of the CD28-mediated proliferation of ex vivo polyclonal CD4$^+$ T cells is ~10^{10} or 33 population doublings (*7*). The limits of CD4$^+$ T cell proliferation in HIV-infected patients have not yet been determined, because 7 of 10 cultures were terminated after 4 to 8 weeks of culture and cell proliferation remained in the exponential phase. However, the observed increase appeared to be substantial, with a geometric mean expansion of 6.7 log units in the two CD4$^+$ cell cultures that were continued to plateau phase (Table 1). The mean percent-

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age of $CD4^+$ T lymphocytes in the expanded cultures was 86% and varied from 39 to 99% (*10*). The antigen receptor diversity of the expanded populations of $CD4^+$ T cells was assessed by flow cytometric measurement of the TCR V_β repertoire in seven patients after 4 to 12 weeks in culture. All patients had diverse V_β family expression similar to the input population of cells (*8*), in a manner consistent with a CD28-mediated proliferation of polyclonal $CD4^+$ lymphocytes.

Consistent with previous results with normal cells (*11*), the cells from the HIV-infected patients secreted large amounts of T helper cell type 1 (T_H1) cytokines after stimulation with immobilized CD3 and CD28 mAbs. Cytokine accumulation was measured by enzyme-linked immunosorbent assay (ELISA) in eight of the cultures (*12*). The range was 18 to 215 ng/ml (mean, 74 ng/ml) for IL-2, 5 to 78 ng/ml (mean, 32 ng/ml) for interferon-γ (IFN-γ), 0.005 to 0.25 ng/ml (mean, 0.09 ng/ml) for IL-4, 0.03 to 0.66 ng/ml (mean, 0.32 ng/ml) for IL-5, and 3 to 10 ng/ml (mean, 6.7 ng/ml) for tumor necrosis factor-α (TNF-α). Secretion of the C-C chemokines by CD8 cells has been shown to mediate a noncytotoxic antiviral effect (*13*). The concentration of the C-C chemokines RANTES, MIP-1α, and MIP-1β in supernatants of cultures of CD4 cells did not vary consistently with CD28 costimulation (*8*), and therefore the presence of these chemokines is unlikely to be sufficient to mediate the CD28 antiviral effect. However, our results do not exclude a necessary role for chemokines in the antiviral effect.

To determine whether limiting dilution by discarding cells during culture might enrich the number of uninfected cells and thus account for the loss of HIV-1–infected cells, we performed large-volume culture by addition of medium without removal of cells. As shown in Table 2, the amount of proviral *gag* DNA and p24 antigen decreased in culture at the same rate, regardless of cell discards. We found that CD28 stimulation routinely permitted large-scale culture, in the absence of cell discards and antiretroviral drugs, to $>10^{10}$ $CD4^+$ T cells from patients with intermediate-stage HIV-1 infection. Thus, these experiments indicated that the frequency of HIV-infected $CD4^+$ T cells decreases during culture with CD28 mAb, and therefore that this antiviral effect cannot be attributed to serial replacement of medium and cells with fresh medium during the cell culture process.

The observed reduction in viral load during proliferation of patient lymphocytes after treatment with immobilized CD28 mAb is in contrast to previous studies that showed that the addition of soluble CD28 mAb to cultures of lymphocytes from HIV-1–infected donors resulted in enhanced HIV-1 expression and that in vitro infection of $CD4^+$ T cells was followed by enhanced HIV-1 production (*14*). To assess whether the mode of CD28 stimulation might be important in determining the extent of HIV-1 replication after in vitro infection, we cultured CD8-depleted PBMCs in the presence of high-titer $HIV\text{-}1_{Ba\text{-}L}$ and activated them with CD3 mAb and either immobilized or soluble CD28 mAb. PHA-stimulated cells and cells stimulated with soluble CD28 mAb developed high concentrations of p24 antigen (Fig. 2A). In marked contrast, the cultures stimulated with immobilized CD28 mAb did not contain detectable concentrations of p24. Cultures stimulated with soluble CD3 and CD28 mAbs developed the highest concentrations of p24 (Fig. 2C), consistent with previous observations (*14*). The differences in HIV-1 p24 concentrations were not the result of differences in the strength of T cell activation, because the increase in the numbers of cells during the experiment was equivalent for all forms of activation (Fig. 2B). Results similar to those shown in Fig. 2 and Table 1 were also observed in experiments in which cell cultures containing highly purified $CD4^+$ T cells were used instead of CD8-depleted PBMCs.

These results indicate that, depending on the mode of CD28 receptor engagement, costimulation can enhance or potently inhibit HIV-1 expression or the susceptibility to HIV-1 infection as assessed by p24 concentration in culture supernatants from $CD4^+$ T cells. To distinguish between these possibilities and to ascertain the stage of this antiviral effect in the life cycle of HIV-1 infection, we stimulated cells with PHA or CD28 mAb for 3 days before infection with high-titer HIV-1 and used quantitative PCR to assess the kinetics of full-length *gag* DNA accumulation (Fig. 3). After 4 hours of exposure to HIV-1, cells previously stimulated with either PHA plus IL-2 or immobilized CD3 mAb plus IL-2 had large amounts of viral *gag* DNA within 12 to 24 hours of culture. In contrast, cells stimulated with immobilized CD3 and CD28 mAbs had background or near-background amounts of

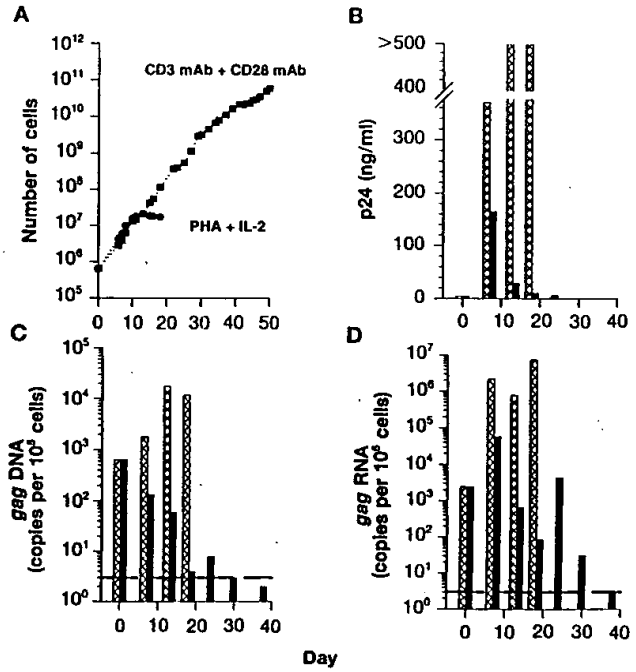

Fig. 1. CD28 mAb–mediated $CD4^+$ T cell proliferation from an HIV-infected patient in the absence of exogenous feeder cells, cytokines, or antiretroviral agents. $CD4^+$ T cells from patient 9 (Table 1) were cultured in either PHA (5 μg/ml, Sigma) plus IL-2 (100 U/ml, Boehringer Mannheim) or beads coated with CD3 and CD28 mAbs, as described below. Cells and supernatants were harvested at 5- to 7-day intervals for analysis of viral burden and cell proliferation. **(A)** Growth curve. The culture stimulated with PHA plus IL-2 was terminated on day 18 because of cytopathic viral effects. **(B)** Concentration of p24 antigen. Cell-free culture supernatants were analyzed for p24 by Coulter ELISA. **(C** and **D)** HIV-1 viral burden. Quantitative measurements of cellular HIV-1 *gag* RNA and proviral DNA were done by PCR with 5×10^6 cells per point, as described below. The horizontal line indicates the lower limit of sensitivity (five copies per 10^5 cells). $CD4^+$ T cells were purified by negative selection [magnetic beads (Dynal) coated with antibodies were used to remove non-CD4 cells (*7*)]. Cells were cultured in plastic tissue culture flasks at 1×10^6 to 2×10^6 cells/ml in RPMI 1640 containing 10% heat-inactivated fetal bovine serum (Hyclone, Logan, Utah), 2 mM L-glutamine, and 20 mM Hepes. Cells were stimulated with immunomagnetic beads (Dynal) that were loaded by tosyl conjugation with equal amounts of CD3 mAb OKT3 and CD28 mAb 9.3 as described (*7*). Beads were added at a ratio of three beads per cell. The cell cultures were monitored by electronic cell sizing (Coulter Counter ZM and Channelyzer 256; Coulter, Hialeah, Florida), and cultures were restimulated with additional beads when the volume of the T cell blasts decreased to <400 fl. No exogenous cytokines or feeder cells were added to the CD28 culture. PCR amplification of β-globin and HIV-1 *gag* with detection by liquid hybridization was used to measure sequential changes in HIV-1 *gag* DNA and RNA during cell culture. Quantitation of HIV-1 *gag* RNA and proviral *gag* DNA in frozen cell pellets was performed as described (*26*).

Table 1. Proliferation, mediated by CD3 and CD28 mAbs, of CD4 cells from HIV-infected donors. PBMCs were obtained from HIV-1–infected persons. The enriched CD4 T cells were cultured with beads coated with CD3 and CD28 mAbs, as described in Fig. 1. Patients had U.S. Centers for Disease Control and Prevention category 2A or 2B infection. The percentage of CD4 cells at culture initiation and at termination and the calculated extent of CD4 cell proliferation are indicated (*25*). Coulter ELISA (Coulter, Hialeah, Florida) was used to detect p24 antigen in culture supernatants at 7- to 14-day intervals; the peak concentration during in vitro culture is indicated. HIV-1 viral burden was determined as described in Fig. 1 at 7- to 14-day intervals (*8*); initial and final values are shown. Culture treatment code: A, azidothymidine (AZT, 1 μM); D, 2′,3′-dideoxyinosine (ddI, 5 μM); N, Nevirapine (0.5 μM). For CD4 cell proliferation, cultures on patients 1 to 8 are submaximal expansions and were terminated while cell proliferation remained in the exponential phase, whereas cultures on patients 9 and 10 were carried to maximum expansion with addition of exogenous IL-2. ND, not done.

Patient	CD4 count (cells/mm³)	Culture treatment	CD4 cells (%)		CD8 cells (%)		CD4 cell proliferation		Peak p24 concentration (ng/ml)	*gag* RNA (copies per 10^5 cells)		*gag* DNA (copies per 10^5 cells)	
			Initial	Final	Initial	Final	log (number of cells)	Days		Initial	Final	Initial	Final
1	554	A, D	93.2	97.1	1.9	1.2	3.7	35	<0.1	55	<3	<3	<3
2	433	A, D	93.2	95.8	2.2	2.8	2.8	29	<0.1	224	<3	190	20
3	430	A, D, N	93.4	97.2	2.1	3.0	4.0	34	<0.1	7,090	15	1470	51
4	445	None	92.3	98.7	2.3	5.2	3.1	28	<0.1	147	23	128	37
5	355	None	ND	95.8	ND	2.9	4.5	40	<0.1	8	<3	107	<3
6	384	None	91.7	39	1.6	56.3	3.3	36	<0.1	313	<3	267	<3
7	466	None	93.6	95.8	1.6	2.6	3.6	28	<0.1	64	<3	57	<3
8	500	None	82.8	67.7	10.0	35.9	2.2	28	<0.1	267	<3	309	<3
9	401	None	ND	97.8	ND	2.0	6.9	71	0.17	2,448	<10	636	<5
10	413	None	64.1	70.2	2.9	18.7	6.5	61	<0.1	14,037	20	614	<5

Table 2. Outgrowth of uninfected CD4 cells from an HIV-infected donor during large-volume culture. CD4 cells (containing <0.5% CD8 cells) were obtained by two sequential rounds of magnetic bead immunodepletion of non-CD4 cells from an HIV-infected donor (CD4 count, 393 cells/mm³). The cells were cultured in a 3-liter gas-permeable bag (Baxter) with stimulation by CD3 and CD28 mAbs for 17 days, during which time there were no cell discards (Bag). Alternatively, cells were grown in companion cultures in T25 flasks, with discards as necessary to maintain cells during feeding with fresh medium (Flask). No antiretroviral agents were added to the cultures. Cell samples were collected for quantitative PCR of *gag* DNA, and culture supernatants were tested for p24 content on the indicated days of culture. The extent of cell proliferation was nearly identical in the two culture conditions; bag cells increased from 30×10^6 to 6.7×10^9 cells, and flask cells increased from 5×10^6 to 2.3×10^9 cells.

Day	Culture conditions	*gag* DNA (copies per 10^5 cells)	p24 (pg/ml)
0	Medium only	802	<50
7	Bag	100	565
	Flask	136	667
11	Bag	110	96
	Flask	72	99
14	Bag	19	<50
	Flask	28	<50
17	Bag	21	<50
	Flask	52	<50

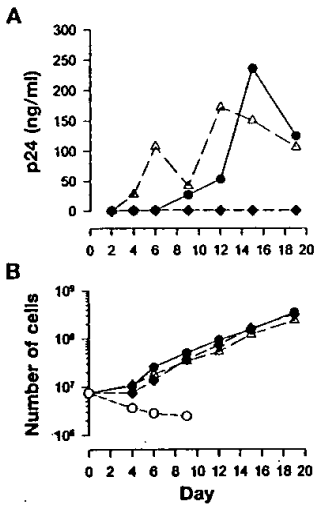

Fig. 2. CD28 can potentiate or inhibit HIV-1 infection of CD4 cells in vitro. (**A** and **B**) CD8-depleted PBMCs were cultured for 2 days in PHA (5 μg/ml) plus IL-2 (100 U/ml) (●), soluble CD3 mAb (100 ng/ml) plus IL-2 (100 U/ml) plus soluble CD28 mAb (1 μg/ml) (Δ), beads coated with CD3 and CD28 mAbs (◆), or medium only (○) as described in Fig. 1. HIV-$1_{Ba\text{-}L}$ (*27*) was also added on day 0 at 2666 times the median tissue culture infectious dose ($TCID_{50}$) per 1×10^6 cells. On day 2, virus was washed out of all cultures, and cells were replated in conditioned media obtained from companion cultures of uninfected cells. Cells were harvested after a further 2 to 17 days of culture, with fresh media added as necessary to maintain the cells at a concentration of 1×10^6 to 2×10^6 cells/ml. Cell-free supernatants were harvested at 2- to 3-day intervals and p24 concentrations were determined by ELISA. Cell counts are averages of two measurements with a Coulter Counter ZM. (**C**) CD8-depleted PBMCs were cultured for 2 days in soluble CD3 mAb (100 ng/ml) plus soluble CD28 mAb (1 μg/ml) (○), soluble CD3 mAb (100 ng/ml) plus IL-2 (100 U/ml) (□), soluble CD3 mAb (100 ng/ml) plus soluble CD28 mAb (1 μg/ml) plus IL-2 (100 U/ml) (Δ), soluble CD3 mAb (100 ng/ml) plus beads coated with CD28 mAb (▼), or beads coated with CD3 and CD28 mAbs (◆). HIV-$1_{Ba\text{-}L}$ was also added on day 0 at 5000 $TCID_{50}$ per 1×10^6 cells. After 2 days, cultures were washed to remove virus and supernatants were harvested as described above.

gag DNA at all time points assessed (Fig. 3A). The antiviral effect could not be attributed to CD3 mAb stimulation alone, as CD4 cells stimulated with immobilized CD3 mAb were infected after exposure to HIV-1, whereas cells exposed to beads coated with CD3 and CD28 mAbs were protected from infection (*8*, *15*). There was specificity for the CD28-mediated antiviral effect because CD4 cells stimulated with beads coated with CD3 and MHC I mAbs were susceptible to infection, whereas beads coated with CD3 and CD28 mAbs rendered the cells resistant to infection (Fig. 3D). Phosphorimager analysis indicated that the amount of *gag* PCR product in cells stimulated with CD3 and CD28 mAbs was less than one-hundredth the amount in cells stimulated with PHA and IL-2. A similar difference was observed when cells infected with HIV-$1_{US\text{-}1}$ were stimulated with PHA and IL-2 or with PHA, IL-2, and immobilized CD28 mAb, which indicated that CD28 stimulation could prevent HIV-1 infection in the context of stimulation with PHA and IL-2 (Fig. 3E). Thus, CD28 costimulation conferred marked resistance to HIV-1 infection. The effect did not appear to depend on the strain of virus used

for infection (9), which is consistent with the ability of CD28 costimulation to increase the number of $CD4^+$ cells from multiple patients infected with HIV-1.

The anti-infective effect of CD28 occurred early in the life cycle of HIV-1 infection, before integration. The mechanism was shown to be multifactorial and to involve a prominent protective effect of CD28 stimulation against HIV-1 infection as well as a proliferative advantage of HIV-uninfected $CD4^+$ T cells over HIV-infected cells. Our initial studies indicate that CD4 receptor function remains normal in cells stimulated with CD3 and CD28 mAbs, which suggests that the HIV-1–resistant state is downstream of HIV-1 binding. It is possible that the permissive and inhibitory forms of CD28 costimulation reflect differential signal transduction (*16*) and that distinct forms of signal transduction confer HIV-1–susceptible or HIV-1–resistant states. As was noted above, the CD28-mediated antiviral effect we describe appears to be distinct from that previously described by Levy and others (*17*).

Our results demonstrate that proliferation of polyclonal HIV-1–uninfected $CD4^+$ T cells from HIV-infected donors is possible, and that CD28 stimulation provides a selective proliferative advantage to subsets of cells that do not support infection. Alternatively, CD28 stimulation could inhibit HIV-1 replication or induce death of the HIV-infected cells. With regard to uninfected $CD4^+$ T cells, there are several possible mechanisms whereby CD28 stimulation could provide a selective proliferative advantage. First, CD28 induces the proliferation of a subset of cells that does not include HIV-infected $CD4^+$ T cells. HIV-1 resides primarily in polyclonal $CD4^+$ cells with the memory phenotype (*1*, *18*). The selective induction of proliferation of naive $CD4^+$ cells by CD28 mAb is unlikely to explain our results, because stimulation with CD3 and CD28 mAbs has been shown to induce the proliferation of essentially all $CD4^+$ cells from normal individuals, including memory and naive subsets (*7*, *19*). Second, in some patients, $CD4^+$ cells of the TCR $V_\beta 12$ family are preferentially infected with HIV-1, and under certain culture conditions HIV-1 replicates 10 to 100 times as efficiently in $CD4^+$ $V_\beta 12$ cells as in control V_β cells (*20*). This possibility was excluded because we did not find a preferential selection against cells that express $V_\beta 12$ in the four cultures that were examined before and after CD28-mediated $CD4^+$ cell proliferation (*21*). Third, HIV-positive individuals have an increasing fraction of $CD28^-$ T cells (*22*), and it is likely that CD28 stimulation confers a proliferative advantage to the $CD28^+$ T cells. It is possible that the viral burden is disproportionately represented in the $CD28^-$ cells. We have also considered that CD28 may selectively stimulate the differentiation of CD4 cells that do not support HIV-1 infection. For example, other investigators have concluded that HIV-1 preferentially replicates in $CD4^+$ T cells producing T_H2-type cytokines (T_H2 and T_H0 cells) (*23*). Further studies will be required to test these notions.

The culture of large numbers of $CD4^+$ T cells from HIV-infected patients has proven difficult. In one study, successful $CD4^+$ T cell proliferation in a subset of HIV-infected patients required the addition of multiple antiretroviral agents to cell culture medium to prevent viral expression (*24*). However, clinical utility was limited by drug-resistant virus breakthrough and a requirement for allogeneic feeder cells to restimulate lymphocytes. Several therapeutic approaches to HIV-1 infection might be facilitated by our results. Ex vivo proliferation of $CD4^+$ T cells may permit immune reconstitution and vaccine therapies involving autologous transfusions of polyclonal or antigen-specific $CD4^+$ T cells into patients. Moreover, autologous transfusions of CD4 lymphocytes might provide the immunologic help necessary to sustain $CD8^+$ T cell function. We have found that although CD28 stimulation can prevent HIV-1 infection and expression, it supports high transduction efficiencies with Moloney leukemia virus–based retroviral vectors (9); hence, culture systems that use CD28 costimulation might be an efficacious way to generate $CD4^+$ T cells for gene therapy as well as immunotherapy. Our results demonstrate a potent CD28-mediated antiviral effect in patients with intermediate-stage HIV infection. Preliminary results from a limited number of patients indicate that the antiviral effect may be less potent in late-stage HIV infection, even though CD28 costimulation still enhances $CD4^+$ T cell proliferation. Finally, our results indicate that in vivo manipulation of CD28 interaction with B7 counterreceptors has the potential to enhance $CD4^+$ T cell proliferation and prevent or limit HIV-1 viral spread in patients.

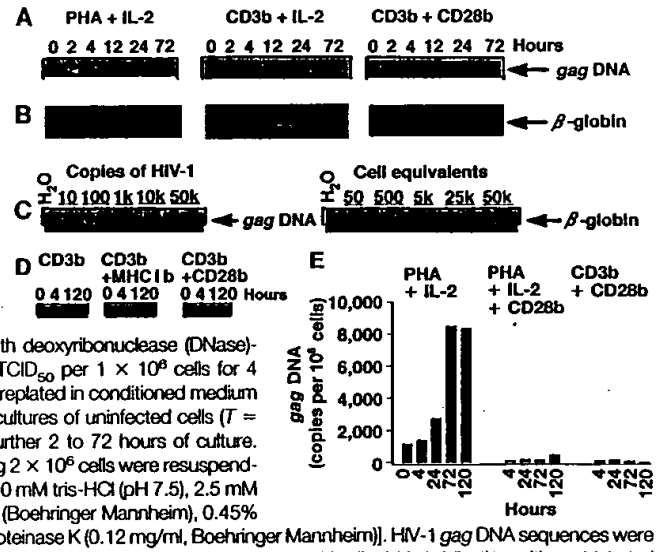

Fig. 3. Immobilized CD28 mAb prevents HIV-1 infection of CD4 cells, as assessed by PCR analysis of HIV-1 *gag*. (**A** and **B**) $CD4^+$ T cells were cultured for 3 days in PHA (5 μg/ml) plus IL-2 (100 U/ml), beads coated with CD3 mAb (CD3b) plus IL-2 (100 U/ml), or beads coated with CD3 and CD28 mAbs (CD3b + CD28b). The cells were collected and incubated with deoxyribonuclease (DNase)-treated HIV-$1_{Ba\text{-}L}$ at 7000 $TCID_{50}$ per 1×10^6 cells for 4 hours, washed three times, replated in conditioned medium obtained from companion cultures of uninfected cells ($T = 0$), and harvested after a further 2 to 72 hours of culture. Frozen cell pellets containing 2×10^6 cells were resuspended in 200 μl of lysis buffer [10 mM tris-HCl (pH 7.5), 2.5 mM $MgCl_2$, 0.45% Triton X-100 (Boehringer Mannheim), 0.45% Tween 20 (Bio-Rad), and proteinase K (0.12 mg/ml, Boehringer Mannheim)]. HIV-1 *gag* DNA sequences were amplified as described (*26*). The amplified products were detected by liquid hybridization with end-labeled oligonucleotide probes, followed by gel electrophoresis. PCR products were quantitated as described (26) with a Molecular Dynamics Phosphorimager. (**C**) Standardization of the PCR products was achieved by parallel amplification of a series of plasmid external control templates. (**D**) CD4 T cells were cultured in IL-2–containing medium as described in (A) and (B) by stimulation with beads coated with CD3 mAb, with CD3 and MHC I mAbs (CD3b + MHC I b), or with CD3 and CD28 mAbs. (MHC I mAb W6/32, which binds to the framework region of HLA class I A, B, and C molecules, served as an isotype and binding control for CD28 mAb 9.3.) Samples were analyzed for *gag* DNA at 0, 4, and 120 hours. (**E**) Immobilized CD28 mAb prevents HIV infection of PHA-stimulated CD4 blasts. $CD4^+$ T cells (95% purity) were cultured for 3 days in medium containing PHA (5 μg/ml) plus IL-2 (100 U/ml), PHA (5 μg/ml) plus IL-2 (100 U/ml) plus beads coated with CD28 mAb, or beads coated with CD3 and CD28 mAbs. The cells were collected and infected with DNase-treated HIV-1_{US1} isolate (*27*) at 1.5×10^3 $TCID_{50}$ per 10^6 cells. Cells were collected for PCR analysis after a further 4 to 120 hours of culture. HIV-1 *gag* and β-globin were quantitated from frozen cell pellets as described above.

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10. Culture with CD3 and CD28 mAbs provides a proliferative advantage for $CD4^+$ over $CD8^+$ lymphocytes with uninfected donors (*7*) [R. Costello *et al.*, *Eur. J. Immunol.* **23**, 608 (1993)]. However, with HIV-infected donors, the proportion of $CD28^-$ lymphocytes is greater and there are more $CD8^+$ lymphocytes in the input culture, so that further enrichment steps would be required to obtain pure $CD4^+$ lymphocytes at culture termination.
11. T_H1 cytokines predominate in cell cultures when immobilized CD28 mAb is used for stimulation (*7*). In contrast, when soluble CD28 mAb is used for cell culture, T_H2 cytokines may predominate [C. L. King, R. J. Stupi, N. Craighead, C. H. June, G. Thyphronitis, *Eur. J. Immunol.* **25**, 587 (1995); W. Holter, O. Majdic, F. S. Kalthoff, W. Knapp, *ibid.* **22**, 2765 (1992); T. van der Pouw Kraan, R. de Jong, L. Aarden, *ibid.* **23**, 1 (1993)]. In vivo, CD28-B7 blockade after alloantigenic challenge inhibits T_H1 cytokines but spares T_H2 cytokines [M. H. Sayegh *et al.*, *J. Exp. Med.* **181**, 1869 (1995)]. Thus, the mode of CD28 stimulation may affect T_H1 and T_H2 development [J. A. Bluestone, *Immunity* **2**, 555 (1995); C. B. Thompson, *Cell* **81**, 979 (1995)].
12. Cytokine secretion was tested in cultures from eight patients in Table 1 after 10 to 20 days in culture. Cells were collected and washed, and the extent of cytokine secretion upon restimulation was determined by placing the cells into fresh medium with beads coated with CD3 and CD28 mAbs for 24 hours. Cell-free culture supernatants were analyzed for IL-2, IFN-γ, IL-4, IL-5, and TNF-α by ELISA with the use of commercially available kits [T Cell Diagnostics, Woburn, MA (IL-2); R&D Systems, Minneapolis (TNF-α, IL-4, and IL-5); Endogen, Boston (IFN-γ)].
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15. CD4 cells have a hierarchy of permissiveness to HIV-1 infection that is determined by the mode of stimulation with various mitogens. In most donors, the susceptibility to HIV-1 infection is [soluble CD3 mAb, soluble CD28 mAb] → [concanavalin A, PHA] → [immobilized CD3 mAb, soluble CD28 mAb] → [immobilized CD3 mAb, immobilized CD28 mAb]. The increased susceptibility of CD4 cells to HIV infection after stimulation with soluble antibody has been noted previously (*14*).
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17. We have considered whether the antiviral effect mediated by CD28 costimulation might be a soluble factor or factors previously described by others [C. M. Walker, D. J. Moody, D. P. Stites, J. A. Levy, *Science* **234**, 1563 (1986)]. Given that the factor first described by Levy is secreted by CD8 cells and suppresses viral replication late in the viral life cycle, it is likely that the CD28 effect we have described is distinct. First, as shown in Table 2, the effect mediated by CD28 requires <0.6% CD8 cells, and thus it is either independent of CD8 T cells or dependent on trace amounts of CD8 cells. Second, the CD28 effect acts early in the viral life cycle to prevent infection of CD4, whereas the effect first described by Levy is reported to act late in the viral life cycle to suppress infection. Third, Cocchi *et al.* (*13*) have reported that chemokines are the major mediator of the CD8 antiviral effect, and we have found that the secretion of C-C chemokines is not dependent on CD28 stimulation (*9*).
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21. TCR $V_\beta12$ analysis was done by flow cytometry on four paired cultures of CD4 cells from patients shown in Table 1 before and after 3 to 5 weeks in culture. The $V_\beta12$ population was 1.0 ± 0.4% on day 0 and 2.8 ± 1.4% at the end of the culture period (mean ± SD, $P = 0.18$).
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Solution Structure of a Two-Base DNA Bulge Complexed with an Enediyne Cleaving Analog

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Nucleic acid bulges have been implicated in a number of biological processes and are specific cleavage targets for the enediyne antitumor antibiotic neocarzinostatin chromophore in a base-catalyzed, radical-mediated reaction. The solution structure of the complex between an analog of the bulge-specific cleaving species and an oligodeoxynucleotide containing a two-base bulge was elucidated by nuclear magnetic resonance. An unusual binding mode involves major groove recognition by the drug carbohydrate unit and tight fitting of the wedge-shaped drug in the triangular prism pocket formed by the two looped-out bulge bases and the neighboring base pairs. The two drug rings mimic helical DNA bases, complementing the bent DNA structure. The putative abstracting drug radical is 2.2 ± 0.1 angstroms from the pro-*S* H5′ of the target bulge nucleotide. This structure clarifies the mechanism of bulge recognition and cleavage by a drug and provides insight into the design of bulge-specific nucleic acid binding molecules.

Bulged structures (regions of unpaired bases) in nucleic acids have been the subject of intense interest (*1*), because they have been implicated as binding motifs for regulatory proteins in viral replication (*2*), as targets for repair enzymes in imperfect homologous recombination (*3*), as products of slipped mispairing in the replication of microsatellite DNA (*4*), as intermediates in frameshift mutations (*5*), and as essential elements in naturally occurring antisense RNAs (*6*).

Neocarzinostatin chromophore (NCS chrom) is unusual among the naturally occurring enediyne antibiotics (*7*) in its ability to attack specifically and exclusively a single residue at a two-base bulge of certain DNA sequences under the influence of general base catalysis (*8*). Under the same conditions NCS chrom cleaves the transactivation response element of human immunodeficiency virus type I viral RNA with high specificity at one of its proposed bulge residues (*9*). Further, studies with long single-stranded DNAs, similar to ones found in some DNA viruses, have revealed related binding-cleavage sites located at bulged sites (*10*). This

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APPENDIX C

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Antiviral Effect and Ex Vivo CD4$^+$ T Cell Proliferation in HIV-Positive Patients as a Result of CD28 Costimulation

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Because stimulation of CD4$^+$ lymphocytes leads to activation of human immunodeficiency virus–type 1 (HIV-1) replication, viral spread, and cell death, adoptive CD4$^+$ T cell therapy has not been possible. When antigen and CD28 receptors on cultured T cells were stimulated by monoclonal antibodies (mAbs) to CD3 and CD28 that had been immobilized, there was an increase in the number of polyclonal CD4$^+$ T cells from HIV-infected donors. Activated cells predominantly secreted cytokines associated with T helper cell type 1 function. The HIV-1 viral load declined in the absence of antiretroviral agents. Moreover, CD28 stimulation of CD4$^+$ T cells from uninfected donors rendered these cells highly resistant to HIV-1 infection. Immobilization of CD28 mAb was crucial to the development of HIV resistance, as cells stimulated with soluble CD28 mAb were highly susceptible to HIV infection. The CD28-mediated antiviral effect occurred early in the viral life cycle, before HIV-1 DNA integration. These data may facilitate immune reconstitution and gene therapy approaches in persons with HIV infection.

CD4$^+$ T cells contain the major reservoir of HIV-1 in vivo (*1*, *2*). The difficulties involved in inducing proliferation in vitro of autologous CD4$^+$ T cells from patients with HIV-1 infection limit the therapeutic potential for many approaches that involve gene therapy or immune reconstitution of infected persons (*3*). Two obstacles attributed to impaired CD4$^+$ T cell proliferation are a limited clonogenic potential of the uninfected CD4 and CD8 cells and the activation of HIV-1 expression and viral production (*4*). In addition to T cell receptor (TCR) engagement of an antigenic peptide bound to major histocompatibility complex (MHC) receptors, other costimulatory signals are necessary for T cell activation. The most important of the costimulatory signals identified to date is provided by the interaction of CD28 on T cells with its ligands CD80 and CD86 on antigen-presenting cells (*5*). Because CD28 signal transduction can prevent apoptosis in cultures of HIV-infected cells and can induce expression of the *Bcl-X$_L$* cell survival gene (*6*), we tested the hypothesis that costimulation might be limiting in cultures from HIV-infected patients.

We cultured lymphocytes from 10 patients with HIV-1 infection (CD4 counts of 350 to 600 cells/mm^3) in the presence of beads coated with CD3 mAb OKT3 and CD28 mAb 9.3 (Table 1). Cell culture was performed in the absence of exogenous cytokines or feeder cells, as CD28 stimulation provides the necessary costimulus to replace feeder cells (*7*). Figure 1 shows the growth curve of CD4$^+$ T cells from an HIV-infected patient after stimulation by a conventional method [with phytohemagglutinin (PHA) and interleukin-2 (IL-2)] or with CD3 and CD28 mAbs in medium that did not contain antiretroviral agents. In the PHA-stimulated culture, the growth curve revealed an initial exponential expansion and a subsequent plateau phase, resulting in termination on day 18 of the culture (Fig. 1A). This pattern was coincident with increased p24 antigen production and with increased viral burden as measured by a quantitative polymerase chain reaction (PCR) for cellular HIV-1 *gag* (Fig. 1, B to D). In contrast, when cells were cultured with CD3 and CD28 mAbs, exponential cell proliferation was maintained for 50 days (Fig. 1A). Although there was evidence of modest viral expression early in the culture, as indicated by the concentration of p24 on day 8 (Fig. 1B), viral production and proviral DNA decreased to undetectable amounts in the culture (Fig. 1, C and D). Similar results were obtained whether the starting cell population was peripheral blood mononuclear cells (PBMCs) or purified CD4$^+$ T cells; this finding suggested that the enhanced cell proliferation and antiviral effects in the culture stimulated with CD3 and CD28 mAbs were not dependent on CD8$^+$ T cells or accessory cells (Table 2) (*8*).

A quantitative PCR was used to determine amounts of HIV-1 *gag* DNA and RNA in the cultures of lymphocytes from the 10 HIV-positive patients (Table 1). Culture with CD28 mAb resulted in decreased viral burden in all patient-derived cells, including the cells cultured in the absence of antiretroviral agents. HIV-1 *gag* proviral DNA became undetectable in six of seven cultures from patients that were cultured in the absence of antiretroviral agents, and HIV-1 *gag* RNA became undetectable in five of the seven cultures. Culture supernatants were also sampled for p24 antigen at 7- to 14-day intervals. Antigen was not detected in 9 of the 10 patients; in one patient (patient 9; Table 1 and Fig. 1), decreasing concentrations of p24 antigen with time were detected. Virus-free CD4$^+$ T cell proliferation also occurred even when CD8 cells constituted <1% of the cells (Table 2) (*9*).

The increase in the number of CD4 cells was not significantly different in the presence or absence of a combination of antiretroviral agents in three patients (*8*). In uninfected adult blood donors, the average absolute magnitude of the CD28-mediated proliferation of ex vivo polyclonal CD4$^+$ T cells is ~10^{10} or 33 population doublings (*7*). The limits of CD4$^+$ T cell proliferation in HIV-infected patients have not yet been determined, because 7 of 10 cultures were terminated after 4 to 8 weeks of culture and cell proliferation remained in the exponential phase. However, the observed increase appeared to be substantial, with a geometric mean expansion of 6.7 log units in the two CD4$^+$ cell cultures that were continued to plateau phase (Table 1). The mean percent-

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age of CD4+ T lymphocytes in the expanded cultures was 86% and varied from 39 to 99% (*10*). The antigen receptor diversity of the expanded populations of CD4+ T cells was assessed by flow cytometric measurement of the TCR V_β repertoire in seven patients after 4 to 12 weeks in culture. All patients had diverse V_β family expression similar to the input population of cells (*8*), in a manner consistent with a CD28-mediated proliferation of polyclonal CD4+ lymphocytes.

Consistent with previous results with normal cells (*11*), the cells from the HIV-infected patients secreted large amounts of T helper cell type 1 (T_H1) cytokines after stimulation with immobilized CD3 and CD28 mAbs. Cytokine accumulation was measured by enzyme-linked immunosorbent assay (ELISA) in eight of the cultures (*12*). The range was 18 to 215 ng/ml (mean, 74 ng/ml) for IL-2, 5 to 78 ng/ml (mean, 32 ng/ml) for interferon-γ (IFN-γ), 0.005 to 0.25 ng/ml (mean, 0.09 ng/ml) for IL-4, 0.03 to 0.66 ng/ml (mean, 0.32 ng/ml) for IL-5, and 3 to 10 ng/ml (mean, 6.7 ng/ml) for tumor necrosis factor–α (TNF-α). Secretion of the C-C chemokines by CD8 cells has been shown to mediate a noncytotoxic antiviral effect (*13*). The concentration of the C-C chemokines RANTES, MIP-1α, and MIP-1β in supernatants of cultures of CD4 cells did not vary consistently with CD28 costimulation (*8*), and therefore the presence of these chemokines is unlikely to be sufficient to mediate the CD28 antiviral effect. However, our results do not exclude a necessary role for chemokines in the antiviral effect.

To determine whether limiting dilution by discarding cells during culture might enrich the number of uninfected cells and thus account for the loss of HIV-1–infected cells, we performed large-volume culture by addition of medium without removal of cells. As shown in Table 2, the amount of proviral *gag* DNA and p24 antigen decreased in culture at the same rate, regardless of cell discards. We found that CD28 stimulation routinely permitted large-scale culture, in the absence of cell discards and antiretroviral drugs, to $>10^{10}$ CD4+ T cells from patients with intermediate-stage HIV-1 infection. Thus, these experiments indicated that the frequency of HIV-infected CD4+ T cells decreases during culture with CD28 mAb, and therefore that this antiviral effect cannot be attributed to serial replacement of medium and cells with fresh medium during the cell culture process.

The observed reduction in viral load during proliferation of patient lymphocytes after treatment with immobilized CD28 mAb is in contrast to previous studies that showed that the addition of soluble CD28 mAb to cultures of lymphocytes from HIV-1–infected donors resulted in enhanced HIV-1 expression and that in vitro infection of CD4+ T cells was followed by enhanced HIV-1 production (*14*). To assess whether the mode of CD28 stimulation might be important in determining the extent of HIV-1 replication after in vitro infection, we cultured CD8-depleted PBMCs in the presence of high-titer $HIV\text{-}1_{Bal\text{-}L}$ and activated them with CD3 mAb and either immobilized or soluble CD28 mAb. PHA-stimulated cells and cells stimulated with soluble CD28 mAb developed high concentrations of p24 antigen (Fig. 2A). In marked contrast, the cultures stimulated with immobilized CD28 mAb did not contain detectable concentrations of p24. Cultures stimulated with soluble CD3 and CD28 mAbs developed the highest concentrations of p24 (Fig. 2C), consistent with previous observations (*14*). The differences in HIV-1 p24 concentrations were not the result of differences in the strength of T cell activation, because the increase in the numbers of cells during the experiment was equivalent for all forms of activation (Fig. 2B). Results similar to those shown in Fig. 2 and Table 1 were also observed in experiments in which cell cultures containing highly purified CD4+ T cells were used instead of CD8-depleted PBMCs.

These results indicate that, depending on the mode of CD28 receptor engagement, costimulation can enhance or potently inhibit HIV-1 expression or the susceptibility to HIV-1 infection as assessed by p24 concentration in culture supernatants from CD4+ T cells. To distinguish between these possibilities and to ascertain the stage of this antiviral effect in the life cycle of HIV-1 infection, we stimulated cells with PHA or CD28 mAb for 3 days before infection with high-titer HIV-1 and used quantitative PCR to assess the kinetics of full-length *gag* DNA accumulation (Fig. 3). After 4 hours of exposure to HIV-1, cells previously stimulated with either PHA plus IL-2 or immobilized CD3 mAb plus IL-2 had large amounts of viral *gag* DNA within 12 to 24 hours of culture. In contrast, cells stimulated with immobilized CD3 and CD28 mAbs had background or near-background amounts of

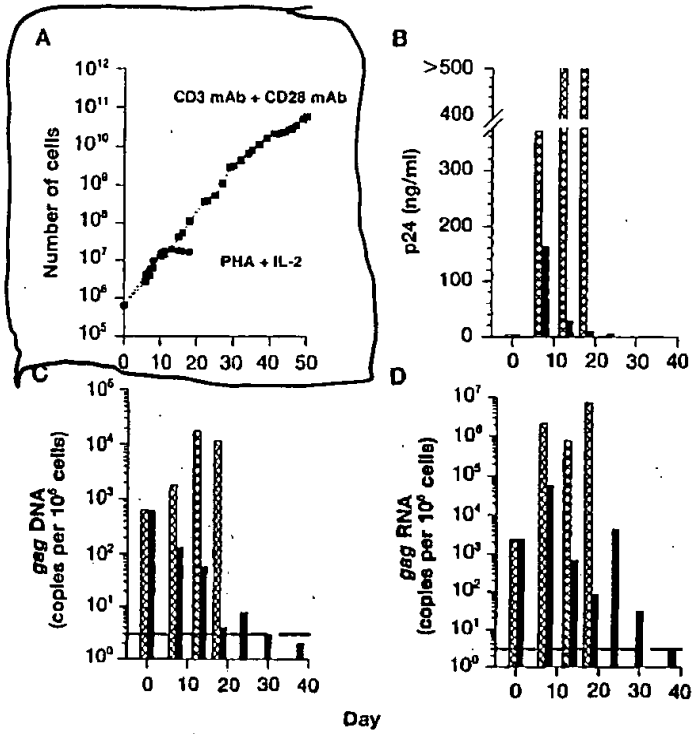

Fig. 1. CD28 mAb–mediated CD4+ T cell proliferation from an HIV-infected patient in the absence of exogenous feeder cells, cytokines, or antiretroviral agents. CD4+ T cells from patient 9 (Table 1) were cultured in either PHA (5 μg/ml, Sigma) plus IL-2 (100 U/ml, Boehringer Mannheim) or beads coated with CD3 and CD28 mAbs, as described below. Cells and supernatants were harvested at 5- to 7-day intervals for analysis of viral burden and cell proliferation. (**A**) Growth curve. The culture stimulated with PHA plus IL-2 was terminated on day 18 because of cytopathic viral effects. (**B**) Concentration of p24 antigen. Cell-free culture supernatants were analyzed for p24 by Coulter ELISA. (**C** and **D**) HIV-1 viral burden. Quantitative measurements of cellular HIV-1 *gag* RNA and proviral DNA were done by PCR with 5×10^6 cells per point, as described below. The horizontal line indicates the lower limit of sensitivity (five copies per 10^5 cells). CD4+ T cells were purified by negative selection (magnetic beads (Dynal) coated with antibodies were used to remove non-CD4 cells (*7*)). Cells were cultured in plastic tissue culture flasks at 1×10^6 to 2×10^6 cells/ml in RPMI 1640 containing 10% heat-inactivated fetal bovine serum (Hyclone, Logan, Utah), 2 mM L-glutamine, and 20 mM Hepes. Cells were stimulated with immunomagnetic beads (Dynal) that were loaded by tosyl conjugation with equal amounts of CD3 mAb OKT3 and CD28 mAb 9.3 as described (*7*). Beads were added at a ratio of three beads per cell. The cell cultures were monitored by electronic cell sizing (Coulter Counter ZM and Channelyzer 256; Coulter, Hialeah, Florida), and cultures were restimulated with additional beads when the volume of the T cell blasts decreased to <400 fl. No exogenous cytokines or feeder cells were added to the CD28 culture. PCR amplification of β-globin and HIV-1 *gag* with detection by liquid hybridization was used to measure sequential changes in HIV-1 *gag* DNA and RNA during cell culture. Quantitation of HIV-1 *gag* RNA and proviral *gag* DNA in frozen cell pellets was performed as described (*26*).

Table 1. Proliferation, mediated by CD3 and CD28 mAbs, of CD4 cells from HIV-infected donors. PBMCs were obtained from HIV-1–infected persons. The enriched CD4 T cells were cultured with beads coated with CD3 and CD28 mAbs, as described in Fig. 1. Patients had U.S. Centers for Disease Control and Prevention category 2A or 2B infection. The percentage of CD4 cells at culture initiation and at termination and the calculated extent of CD4 cell proliferation are indicated (25). Coulter ELISA (Coulter, Hialeah, Florida) was used to detect p24 antigen in culture supernatants at 7- to 14-day intervals; the peak concentration during in vitro culture is indicated. HIV-1 viral burden was determined as described in Fig. 1 at 7- to 14-day intervals (8); initial and final values are shown. Culture treatment code: A, azidothymidine (AZT, 1 μM); D, 2′,3′-dideoxyinosine (ddI, 5 μM); N, Nevirapine (0.5 μM). For CD4 cell proliferation, cultures on patients 1 to 8 are submaximal expansions and were terminated while cell proliferation remained in the exponential phase, whereas cultures on patients 9 and 10 were carried to maximum expansion with addition of exogenous IL-2. ND, not done.

Patient	CD4 count (cells/mm³)	Culture treatment	CD4 cells (%)		CD8 cells (%)		CD4 cell proliferation		Peak p24 concentration (ng/ml)	*gag* RNA (copies per 10^5 cells)		*gag* DNA (copies per 10^5 cells)	
			Initial	Final	Initial	Final	log (number of cells)	Days		Initial	Final	Initial	Final
1	554	A, D	93.2	97.1	1.9	1.2	3.7	35	<0.1	55	<3	<3	<3
2	433	A, D	93.2	95.8	2.2	2.8	2.8	29	<0.1	224	<3	190	20
3	430	A, D, N	93.4	97.2	2.1	3.0	4.0	34	<0.1	7,090	15	1470	51
4	445	None	92.3	98.7	2.3	5.2	3.1	28	<0.1	147	23	128	37
5	355	None	ND	95.8	ND	2.9	4.5	40	<0.1	8	<3	107	<3
6	384	None	91.7	39	1.6	56.3	3.3	36	<0.1	313	<3	267	<3
7	466	None	93.6	95.8	1.6	2.6	3.6	28	<0.1	64	<3	57	<3
8	500	None	82.8	67.7	10.0	35.9	2.2	28	<0.1	267	<3	309	<3
9	401	None	ND	97.8	ND	2.0	6.9	71	0.17	2,448	<10	636	<5
10	413	None	64.1	70.2	2.9	18.7	6.5	61	<0.1	14,037	20	614	<5

Table 2. Outgrowth of uninfected CD4 cells from an HIV-infected donor during large-volume culture. CD4 cells (containing <0.5% CD8 cells) were obtained by two sequential rounds of magnetic bead immunodepletion of non-CD4 cells from an HIV-infected donor (CD4 count, 393 cells/mm³). The cells were cultured in a 3-liter gas-permeable bag (Baxter) with stimulation by CD3 and CD28 mAbs for 17 days, during which time there were no cell discards (Bag). Alternatively, cells were grown in companion cultures in T25 flasks, with discards as necessary to maintain cells during feeding with fresh medium (Flask). No antiretroviral agents were added to the cultures. Cell samples were collected for quantitative PCR of *gag* DNA, and culture supernatants were tested for p24 content on the indicated days of culture. The extent of cell proliferation was nearly identical in the two culture conditions; bag cells increased from 30×10^6 to 6.7×10^9 cells, and flask cells increased from 5×10^6 to 2.3×10^9 cells.

Day	Culture conditions	*gag* DNA (copies per 10^5 cells)	p24 (pg/ml)
0	Medium only	802	<50
7	Bag	100	565
	Flask	136	667
11	Bag	110	96
	Flask	72	99
14	Bag	19	<50
	Flask	28	<50
17	Bag	21	<50
	Flask	52	<50

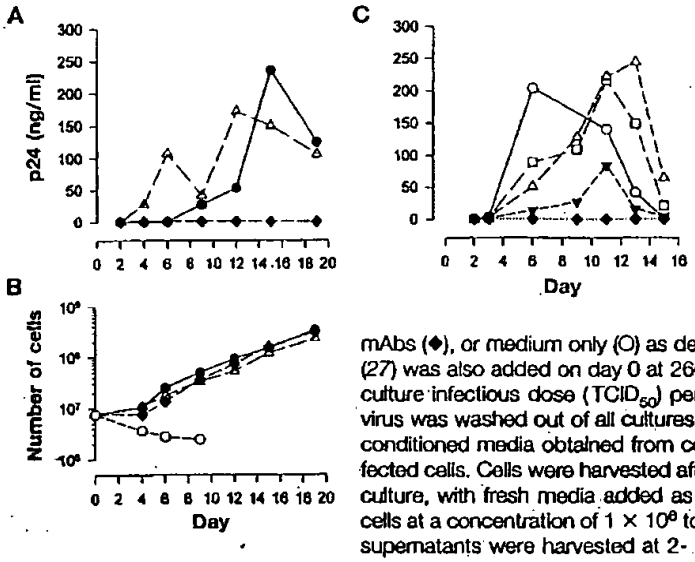

Fig. 2. CD28 can potentiate or inhibit HIV-1 infection of CD4 cells in vitro. (**A** and **B**) CD8-depleted PBMCs were cultured for 2 days in PHA (5 μg/ml) plus IL-2 (100 U/ml) (●), soluble CD3 mAb (100 ng/ml) plus IL-2 (100 U/ml) plus soluble CD28 mAb (1 μg/ml) (Δ), beads coated with CD3 and CD28 mAbs (◆), or medium only (○) as described in Fig. 1. HIV-$1_{Ba\text{-}L}$ (27) was also added on day 0 at 2666 times the median tissue culture infectious dose ($TCID_{50}$) per 1×10^6 cells. On day 2, virus was washed out of all cultures, and cells were replated in conditioned media obtained from companion cultures of uninfected cells. Cells were harvested after a further 2 to 17 days of culture, with fresh media added as necessary to maintain the cells at a concentration of 1×10^6 to 2×10^6 cells/ml. Cell-free supernatants were harvested at 2- to 3-day intervals and p24 concentrations were determined by ELISA. Cell counts are averages of two measurements with a Coulter Counter ZM. (**C**) CD8-depleted PBMCs were cultured for 2 days in soluble CD3 mAb (100 ng/ml) plus soluble CD28 mAb (1 μg/ml) (○), soluble CD3 mAb (100 ng/ml) plus IL-2 (100 U/ml) (□), soluble CD3 mAb (100 ng/ml) plus soluble CD28 mAb (1 μg/ml) plus IL-2 (100 U/ml) (Δ), soluble CD3 mAb (100 ng/ml) plus beads coated with CD28 mAb (▼), or beads coated with CD3 and CD28 mAbs (◆). HIV-$1_{Ba\text{-}L}$ was also added on day 0 at 5000 $TCID_{50}$ per 1×10^6 cells. After 2 days, cultures were washed to remove virus and supernatants were harvested as described above.

gag DNA at all time points assessed (Fig. 3A). The antiviral effect could not be attributed to CD3 mAb stimulation alone, as CD4 cells stimulated with immobilized CD3 mAb were infected after exposure to HIV-1, whereas cells exposed to beads coated with CD3 and CD28 mAbs were protected from infection (*8*, *15*). There was specificity for the CD28-mediated antiviral effect because CD4 cells stimulated with beads coated with CD3 and MHC I mAbs were susceptible to infection, whereas beads coated with CD3 and CD28 mAbs rendered the cells resistant to infection (Fig. 3D). Phosphorimager analysis indicated that the amount of *gag* PCR product in cells stimulated with CD3 and CD28 mAbs was less than one-hundredth the amount in cells stimulated with PHA and IL-2. A similar difference was observed when cells infected with HIV-$1_{US\text{-}1}$ were stimulated with PHA and IL-2 or with PHA, IL-2, and immobilized CD28 mAb, which indicated that CD28 stimulation could prevent HIV-1 infection in the context of stimulation with PHA and IL-2 (Fig. 3E). Thus, CD28 costimulation conferred marked resistance to HIV-1 infection. The effect did not appear to depend on the strain of virus used

for infection (9), which is consistent with the ability of CD28 costimulation to increase the number of $CD4^+$ cells from multiple patients infected with HIV-1.

The anti-infective effect of CD28 occurred early in the life cycle of HIV-1 infection, before integration. The mechanism was shown to be multifactorial and to involve a prominent protective effect of CD28 stimulation against HIV-1 infection as well as a proliferative advantage of HIV-uninfected $CD4^+$ T cells over HIV-infected cells. Our initial studies indicate that CD4 receptor function remains normal in cells stimulated with CD3 and CD28 mAbs, which suggests that the HIV-1–resistant state is downstream of HIV-1 binding. It is possible that the permissive and inhibitory forms of CD28 costimulation reflect differential signal transduction (16) and that distinct forms of signal transduction confer HIV-1–susceptible or HIV-1–resistant states. As was noted above, the CD28-mediated antiviral effect we describe appears to be distinct from that previously described by Levy and others (17).

Our results demonstrate that proliferation of polyclonal HIV-1–uninfected $CD4^+$ T cells from HIV-infected donors is possible, and that CD28 stimulation provides a selective proliferative advantage to subsets of cells that do not support infection. Alternatively, CD28 stimulation could inhibit HIV-1 replication or induce death of the HIV-infected cells. With regard to uninfected $CD4^+$ T cells, there are several possible mechanisms whereby CD28 stimulation could provide a selective proliferative advantage. First, CD28 induces the proliferation of a subset of cells that does not include HIV-infected $CD4^+$ T cells. HIV-1 resides primarily in polyclonal $CD4^+$ cells with the memory phenotype (1, 18). The selective induction of proliferation of naive $CD4^+$ cells by CD28 mAb is unlikely to explain our results, because stimulation with CD3 and CD28 mAbs has been shown to induce the proliferation of essentially all $CD4^+$ cells from normal individuals, including memory and naive subsets (7, 19). Second, in some patients, $CD4^+$ cells of the TCR $V_\beta 12$ family are preferentially infected with HIV-1, and under certain culture conditions HIV-1 replicates 10 to 100 times as efficiently in $CD4^+$ $V_\beta 12$ cells as in control V_β cells (20). This possibility was excluded because we did not find a preferential selection against cells that express $V_\beta 12$ in the four cultures that were examined before and after CD28-mediated $CD4^+$ cell proliferation (21). Third, HIV-positive individuals have an increasing fraction of $CD28^-$ T cells (22), and it is likely that CD28 stimulation confers a proliferative advantage to the $CD28^+$ T cells. It is possible that the viral burden is disproportionately represented in the $CD28^-$ cells. We have also considered that CD28 may selectively stimulate the differentiation of CD4 cells that do not support HIV-1 infection. For example, other investigators have concluded that HIV-1 preferentially replicates in $CD4^+$ T cells producing T_H2-type cytokines (T_H2 and T_H0 cells) (23). Further studies will be required to test these notions.

The culture of large numbers of $CD4^+$ T cells from HIV-infected patients has proven difficult. In one study, successful $CD4^+$ T cell proliferation in a subset of HIV-infected patients required the addition of multiple antiretroviral agents to cell culture medium to prevent viral expression (24). However, clinical utility was limited by drug-resistant virus breakthrough and a requirement for allogeneic feeder cells to restimulate lymphocytes. Several therapeutic approaches to HIV-1 infection might be facilitated by our results. Ex vivo proliferation of $CD4^+$ T cells may permit immune reconstitution and vaccine therapies involving autologous transfusions of polyclonal or antigen-specific $CD4^+$ T cells into patients. Moreover, autologous transfusions of CD4 lymphocytes might provide the immunologic help necessary to sustain $CD8^+$ T cell function. We have found that although CD28 stimulation can prevent HIV-1 infection and expression, it supports high transduction efficiencies with Moloney leukemia virus–based retroviral vectors (9); hence, culture systems that use CD28 costimulation might be an efficacious way to generate $CD4^+$ T cells for gene therapy as well as immunotherapy. Our results demonstrate a potent CD28-mediated antiviral effect in patients with intermediate-stage HIV infection. Preliminary results from a limited number of patients indicate that the antiviral effect may be less potent in late-stage HIV infection, even though CD28 costimulation still enhances $CD4^+$ T cell proliferation. Finally, our results indicate that in vivo manipulation of CD28 interaction with B7 counterreceptors has the potential to enhance $CD4^+$ T cell proliferation and prevent or limit HIV-1 viral spread in patients.

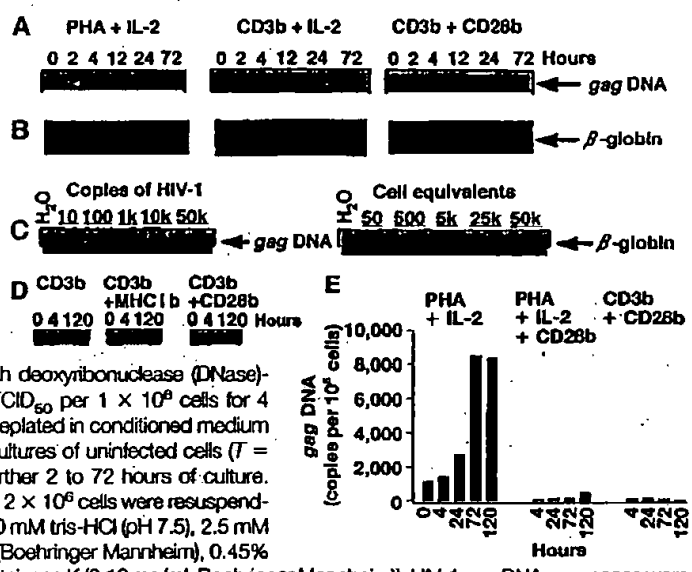

Fig. 3. Immobilized CD28 mAb prevents HIV-1 infection of CD4 cells, as assessed by PCR analysis of HIV-1 *gag*. (A and B) $CD4^+$ T cells were cultured for 3 days in PHA (5 μg/ml) plus IL-2 (100 U/ml), beads coated with CD3 mAb (CD3b) plus IL-2 (100 U/ml), or beads coated with CD3 and CD28 mAbs (CD3b + CD28b). The cells were collected and incubated with deoxyribonuclease (DNase)–treated HIV-$1_{Ba\text{-}L}$ at 7000 $TCID_{50}$ per 1 × 10^6 cells for 4 hours, washed three times, replated in conditioned medium obtained from companion cultures of uninfected cells (T = 0), and harvested after a further 2 to 72 hours of culture. Frozen cell pellets containing 2 × 10^6 cells were resuspended in 200 μl of lysis buffer [10 mM tris-HCl (pH 7.5), 2.5 mM $MgCl_2$, 0.45% Triton X-100 (Boehringer Mannheim), 0.45% Tween 20 (Bio-Rad), and proteinase K (0.12 mg/ml, Boehringer Mannheim)]. HIV-1 *gag* DNA sequences were amplified as described (26). The amplified products were detected by liquid hybridization with end-labeled oligonucleotide probes, followed by gel electrophoresis. PCR products were quantitated as described (26) with a Molecular Dynamics Phosphorimager. (C) Standardization of the PCR products was achieved by parallel amplification of a series of plasmid external control templates. (D) CD4 T cells were cultured in IL-2–containing medium as described in (A) and (B) by stimulation with beads coated with CD3 mAb, with CD3 and MHC I mAbs (CD3b + MHC I b), or with CD3 and CD28 mAbs. (MHC I mAb W6/32, which binds to the framework region of HLA class I A, B, and C molecules, served as an isotype and binding control for CD28 mAb 9.3.) Samples were analyzed for *gag* DNA at 0, 4, and 120 hours. (E) Immobilized CD28 mAb prevents HIV infection of PHA-stimulated CD4 blasts. $CD4^+$ T cells (95% purity) were cultured for 3 days in medium containing PHA (5 μg/ml) plus IL-2 (100 U/ml), PHA (5 μg/ml) plus IL-2 (100 U/ml) plus beads coated with CD28 mAb, or beads coated with CD3 and CD28 mAbs. The cells were collected and infected with DNase-treated HIV-1_{US1} isolate (27) at 1.5 × 10^3 $TCID_{50}$ per 10^6 cells. Cells were collected for PCR analysis after a further 4 to 120 hours of culture. HIV-1 *gag* and β-globin were quantitated from frozen cell pellets as

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10. Culture with CD3 and CD28 mAbs provides a proliferative advantage for $CD4^+$ over $CD8^+$ lymphocytes with uninfected donors (*7*) [R. Costello *et al.*, *Eur. J. Immunol.* 23, 608 (1993)]. However, with HIV-infected donors, the proportion of $CD28^-$ lymphocytes is greater and there are more $CD8^+$ lymphocytes in the input culture, so that further enrichment steps would be required to obtain pure $CD4^+$ lymphocytes at culture termination.
11. T_H1 cytokines predominate in cell cultures when immobilized CD28 mAb is used for stimulation (*7*). In contrast, when soluble CD28 mAb is used for cell culture, T_H2 cytokines may predominate [C. L. King, R. J. Stupi, N. Craighead, C. H. June, G. Thyphronitis, *Eur. J. Immunol.* 25, 587 (1995); W. Holter, O. Majdic, F. S. Kalthoff, W. Knapp, *ibid.* 22, 2765 (1992); T. van der Pouw Kraan, R. de Jong, L. Aarden, *ibid.* 23, 1 (1993)]. In vivo, CD28-B7 blockade after alloantigenic challenge inhibits T_H1 cytokines but spares T_H2 cytokines [M. H. Sayegh *et al.*, *J. Exp. Med.* 181, 1869 (1995)]. Thus, the mode of CD28 stimulation may affect T_H1 and T_H2 development [J. A. Bluestone, *Immunity* 2, 555 (1995); C. B. Thompson, *Cell* 81, 979 (1995)].
12. Cytokine secretion was tested in cultures from eight patients in Table 1 after 10 to 20 days in culture. Cells were collected and washed, and the extent of cytokine secretion upon restimulation was determined by placing the cells into fresh medium with beads coated with CD3 and CD28 mAbs for 24 hours. Cell-free culture supernatants were analyzed for IL-2, IFN-γ, IL-4, IL-5, and TNF-α by ELISA with the use of commercially available kits [T Cell Diagnostics, Woburn, MA (IL-2); R&D Systems, Minneapolis (TNF-α, IL-4, and IL-5); Endogen, Boston (IFN-γ)].
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15. CD4 cells have a hierarchy of permissiveness to HIV-1 infection that is determined by the mode of stimulation with various mitogens. In most donors, the susceptibility to HIV-1 infection is [soluble CD3 mAb, soluble CD28 mAb] → [concanavalin A, PHA] → [immobilized CD3 mAb, soluble CD28 mAb] → [immobilized CD3 mAb, immobilized CD28 mAb]. The increased susceptibility of CD4 cells to HIV infection after stimulation with soluble antibody has been noted previously (*14*).
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17. We have considered whether the antiviral effect mediated by CD28 costimulation might be a soluble factor or factors previously described by others [C. M. Walker, D. J. Moody, D. P. Stites, J. A. Levy, *Science* 234, 1563 (1986)]. Given that the factor first described by Levy is secreted by CD8 cells and suppresses viral replication late in the viral life cycle, it is likely that the CD28 effect we have described is distinct. First, as shown in Table 2, the effect mediated by CD28 requires <0.6% CD8 cells, and thus it is either independent of CD8 T cells or dependent on trace amounts of CD8 cells. Second, the CD28 effect acts early in the viral life cycle to prevent infection of CD4, whereas the effect first described by Levy is reported to act late in the viral life cycle to suppress infection. Third, Cocchi *et al.* (*13*) have reported that chemokines are the major mediator of the CD8 antiviral effect, and we have found that the secretion of C-C chemokines is not dependent on CD28 stimulation (*9*).
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25. The CD4 percentage on day 0 of culture was not obtained for patients 5 and 9 because of limited cell availability; thus, the calculated cell proliferation is the actual cell proliferation without correction for the CD4 count, because only the final CD4 percentage was available.
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28. Supported by Army and Naval Medical Research and Development Commands grant 1437 and NIH grant AI29331. We thank W. Bernstein and J. Lin for intellectual and experimental contributions; C. Thompson for manuscript review; D. Ritchey, S. Perfetto, D. Smoot, N. Craighead, J. Cotte, and S. Barrick for technical assistance; J. D. Malone and J. L. Malone for support and encouragement; clinic patients for blood donation; P. Ganong (Boehringer Ingelheim, Ridgefield, CT) for Nevirapine; and N. Peterson (Ortho Biotech, Raritan, NJ) for CD3 mAb OKT3. The views expressed in this report are those of the authors and do not reflect the official policy or position of the Departments of the Navy and Army, the Department of Defense, or the U.S. government.

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Solution Structure of a Two-Base DNA Bulge Complexed with an Enediyne Cleaving Analog

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Nucleic acid bulges have been implicated in a number of biological processes and are specific cleavage targets for the enediyne antitumor antibiotic neocarzinostatin chromophore in a base-catalyzed, radical-mediated reaction. The solution structure of the complex between an analog of the bulge-specific cleaving species and an oligodeoxynucleotide contalning a two-base bulge was elucidated by nuclear magnetic resonance. An unusual binding mode involves major groove recognition by the drug carbohydrate unit and tight fitting of the wedge-shaped drug in the triangular prism pocket formed by the two looped-out bulge bases and the neighboring base pairs. The two drug rings mimic helical DNA bases, complementing the bent DNA structure. The putative abstracting drug radical is 2.2 ± 0.1 angstroms from the pro-S H5′ of the target bulge nucleotide. This structure clarifies the mechanism of bulge recognition and cleavage by a drug and provides insight into the design of bulge-specific nucleic acid binding molecules.

Bulged structures (regions of unpaired bases) in nucleic acids have been the subject of intense interest (*1*), because they have been implicated as binding motifs for regulatory proteins in viral replication (*2*), as targets for repair enzymes in imperfect homologous recombination (*3*), as products of slipped mispairing in the replication of microsatellite DNA (*4*), as intermediates in frameshift mutations (*5*), and as essential elements in naturally occurring antisense RNAs (*6*).

Neocarzinostatin chromophore (NCS chrom) is unusual among the naturally occurring enediyne antibiotics (*7*) in its ability to attack specifically and exclusively a single residue at a two-base bulge of certain DNA sequences under the influence of general base catalysis (*8*). Under the same conditions NCS chrom cleaves the transactivation response element of human immunodeficiency virus type I viral RNA with high specificity at one of its proposed bulge residues (*9*). Further, studies with long single-stranded DNAs, similar to ones found in some DNA viruses, have revealed related binding-cleavage sites located at bulged sites (*10*). This

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APPENDIX D

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CD3-mediated activation of tumor-reactive lymphocytes from patients with advanced cancer

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Lymphocytes from blood or tumors of patients with advanced cancer did not proliferate and produced very low levels of tumor necrosis factor and IFN-γ when cultured with autologous tumor cells. Proliferation and lymphokine production dramatically increased in the presence of beads conjugated with mAbs to CD3 plus mAbs to CD28 and/or CD40, and the lymphocytes destroyed the tumor cells. Expression density of CD3 concomitantly increased from low to normal levels. Furthermore, beads providing a CD3 signal (in combination with CD28 or CD28 plus CD40) gave partial protection against the inhibitory effect of transforming growth factor type β1 on lymphocyte proliferation and production of tumor necrosis factor and IFN-γ. MHC class I-restricted cytolytic T cells lysing autologous tumor cells in a 4-h Cr^{51} release assay were generated when peripheral blood leukocytes were activated in the presence of autologous tumor cells and anti-CD3/CD28 or anti-CD3/CD28/CD40 beads. Experiments performed in a model system using anti-V-β1 or anti-V-β2 mAbs to activate subsets of T cells expressing restricted T cell receptor showed that lymphocytes previously activated by anti-V-β can respond to CD3 stimulation with vigorous proliferation and lymphokine production while retaining their specificity, also in the presence of transforming growth factor type β1. Our results suggest that T lymphocytes from cancer patients can proliferate and form Th1 type lymphokines in the presence of autologous tumor cell when properly activated, and that antigen released from killed tumor cells and presented by antigen-presenting cells in the cultures facilitates the selective expansion of tumor-directed, $CD8^+$ cytolytic T cells.

immunotherapy | tumor vaccines | tumor immunity | transforming growth factor type β1

Immune responses do not protect against most cancers, although data were published in the 1960s indicating that the immune systems of cancer patients recognize antigens that can be targets for tumor destruction (1). Recent evidence for immunogenic, tumor-associated antigens includes the demonstration, with tetramer technology, that lymphocytes from melanoma patients recognize tumor epitopes (2), the finding of IgG antitumor antibodies by using the SEREX technique (3), and the generation of cytolytic T cells (CTL) to a large variety of tumor epitopes (4, 5). Most likely, the failure of immunological mechanisms to prevent tumor formation is due to mechanisms normally protecting against autoimmunity. For example, most neoplastic (like most normal) cells do not express key costimulatory molecules and are able to "sneak through" immunological control until their antigens have been taken up and processed by "professional" antigen-presenting cells (6–8). Furthermore, tumors make immunosuppressive factors, as can lymphoid cells in response to tumor antigens (9). Members of the transforming growth factor (TGF) type β1 family (9–15) are particularly important in this regard. Reflecting the immunosuppressed state, molecules involved in T cell signaling are down-regulated among lymphocytes from blood or tumors derived from tumor-bearing animals and human patients (16–18). Unless the immunosuppression can be overcome, it is unlikely that tumor vaccination or adoptive transfer of immune T lymphocytes will have a major impact in patients with metastatic cancer.

We now show that lymphocytes from patients with advanced cancer can proliferate, produce high levels of tumor necrosis factor (TNF) and IFN-γ, and generate tumor-destructive CTL in the presence of autologous tumor cells after polyclonal activation, via CD3, and costimulatory signal(s), via CD28, alone or together with CD40. We further show that lymphocytes stimulated via CD3 plus costimulatory signals become relatively resistant to inhibition by TGF-β1. These data are supported by experiments performed in a model system where anti-V-β1 and anti-V-β2 are used as surrogate antigens to which responses are induced or recalled by using the respective specific mAbs.

Materials and Methods

Patient Material. Tumors were obtained at surgery or from malignant effusions (mostly ascites) of patients with stage IV carcinomas. Most studies were performed with eight patients, five of whom (1OV, 3OV, 8OV, 44OV, 48OV) had ovarian carcinoma, two (1C, 22C) had colon carcinoma, and one (1HN) had a head and neck carcinoma. Cells from an ovarian carcinoma line, 4007, also were used.

Preparation of Tumor and Blood Samples. Solid tumors were suspended in medium, and fluids were removed from effusions after which the cells were resuspended. Erythrocytes were removed by Ficoll-Hypaque (Amersham Pharmacia), and a Percoll gradient (Sigma) was used to separate tumor cells from tumor-infiltrating lymphocytes (TIL). Lymphocyte samples were used directly or stored in liquid nitrogen for later use. Tumor samples were explanted to establish cell cultures. Peripheral blood leukocytes (PBL) were purified by using Ficoll-Hypaque. In the initial experiments, $CD8^+$ T lymphocytes (>90% pure) were positively selected from TIL by using VarioMac magnetic beads (Miltenyi Biotech, Auburn, CA). For all other experiments, lymphoid cell populations containing T lymphocytes, monocytes, and B cells were used.

Combination of Lymphocytes and Tumor Cells. In the initial experiments, five lymphocytes were added per tumor cell, after which the mixtures were incubated at 37°C in Costar (3513) 12-well plates (Corning) with RPMI medium (GIBCO) and 10% FCS (Atlanta Biological, Norcross, GA). They were followed by experiments in which PBL or TIL were cultured with or without autologous tumor cells in the presence of magnetic beads (Dynal, Lake Success, NY) and conjugated, using a published technique (19, 20), with mAbs to CD3, CD28, and/or CD40; beads not conjugated with mAb (or

Abbreviations: CTL, cytolytic T cells; FACS, fluorescence-activated cell sorting; PBL, peripheral blood leukocytes; TGF, transforming growth factor; TIL, tumor-infiltrating lymphocytes; TNF, tumor necrosis factor.

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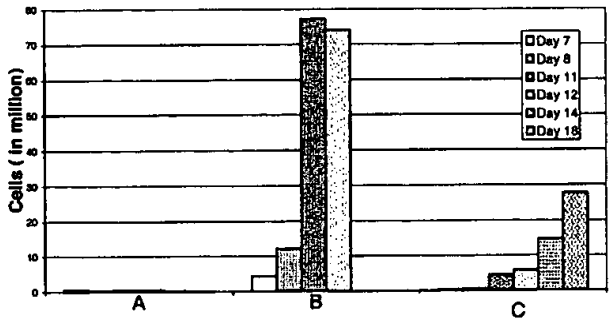

Fig. 1. Proliferation of *in vitro* expanded TILs from 44 OV. (*A*) Autologous tumor, control beads (similar results without tumor). (*B*) Autologous tumor, anti-CD3/CD28/CD40-conjugated beads. (*C*) No tumor, anti-CD3/CD28/CD40-conjugated beads.

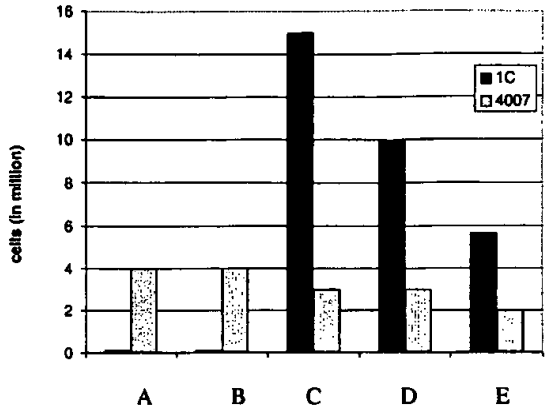

Fig. 2. Proliferation of PBL from 1C after *in vitro* activation in the presence of autologous or allogeneic 4007 mismatched ovarian carcinoma cells. (*A*) Control beads. (*B*) Anti-CD28/CD40-conjugated beads. (*C*) Anti-CD3/CD28-conjugated beads. (*D*) Anti-CD3/CD28/CD40-conjugated beads. (*E*) Anti-CD3/CD40-conjugated beads.

with an irrelevant mAb) were used as controls. The mAbs were 64.1 (21), 9.3 (21), and G28.5 (22), which, respectively, stimulate lymphocytes polyclonally (anti-CD3), costimulate them (anti-CD28), or activate antigen-presenting cells (anti-CD40). When autologous tumor cells were used, cells (40,000–75,000/well) were first attached by overnight incubation to Costar 24-well plates containing 2 ml Iscove's modified Dulbecco medium with 10% FBS. mAb-conjugated beads (3×10^6/ml) were then added, followed by lymphocytes (10^6/ml) in RPMI with 10% FBS. The plates were incubated at 37°C in a 6% CO_2 in air atmosphere for 4–5 days. The beads then were removed using a magnet, and the lymphocytes were placed in new wells in medium containing 10 units/ml of IL-2 (Roche Molecular Biochemicals) and moved into flasks when their concentration had reached $\approx 2 \times 10^6$ cells/ml. Cultures were observed for evidence of tumor cell destruction. Lymphocyte proliferation was determined by counting. Media were sampled to measure production of TNF and/or IFN-γ, which was assayed with WEHI cells (23) and an ELISA (IFN-γ ELISA, EH-IFNG, Endogen, Woburn, MA), respectively. TGF-β1 was purchased from Sigma. In all experiments using TGF-β1, the molecule remained in the cultures, also after removal of mAb-conjugated beads.

CTL Assays. Classical 4-h ^{51}Cr release assays were performed. To characterize the effector cells, experiments were done to inhibit cytotoxicity by addition of mAb w6/32 (10 μg/ml), which recognizes a MHC class I framework determinant (Research Diagnostics, Flanders, NJ). mAbs to the natural killer markers CD16 and CD56 (Beckman Coulter), anti-CD8 mAb HIT8a (PharMingen), and anti-integrin-β2 (CD18) mAb 60.3 (24) also were used.

Fluorescence-Activated Cell Sorting (FACS) Analysis of Lymphocytes. Density of CD expression was evaluated by FACS (Epics XL, Coulter), using phycoerythrin-labeled mAb and counting cells as positive when they had a preset minimum brightness. To investigate whether an increased density of CD3 expression after *in vitro* activation of lymphocytes was due to the selective proliferation of cells with originally high CD3 expression, PBL harvested from cancer patients were labeled with the dye CFDA (Molecular Probes). Subsequently, they were cultured in the presence of anti-CD3/CD28/CD40 beads for 5 days, after which the beads were removed and the lymphocytes were expanded in medium containing 10 units IL-2/ml. At two time points after removal of the beads (4 h and 3 days) FACS analysis was performed, in which cells were analyzed for labeling by CFDA and expression of CD3. Labeled lymphocytes that had been cultured with control beads were studied for comparison.

Use of a Model System with Anti-V-β1 and Anti-V-β2 as Surrogate Antigens. Experiments were performed to investigate the effect of stimulation of PBL from healthy adult donors (one donor in each experiment) on the proliferation and lymphokine production of naïve lymphocytes and on lymphocytes activated by mAbs to V-β1 and V-β2 (Beckman Coulter). The well bottoms of a culture plate were coated with 1 μg/ml of anti-V-β1 or anti-V-β2 by incubation at 4°C overnight. Subsequently, PBL (2×10^6/well) were added, either together with control beads or beads conjugated with anti-CD3/anti-CD28/anti-CD40 mAbs. After 4–5 days of coculture, beads were removed and lymphocytes were expanded in medium supplemented with 10 units IL-2/ml. The number of lymphocytes/well was counted, and production of TNF and IFN-γ was measured. Lymphocytes were analyzed by FACS for expression of CD3, V-β1, and V-β2.

A second round of stimulation then was carried out to study the effects of stimulation by anti-V-β and/or mAb-coated beads on sensitized lymphocytes. In these experiments, lymphocytes sensitized in the presence of either anti-V-β were cultured for 5 days in the presence of the same or a different, immobilized anti-V-β, with or without beads conjugated with anti-CD3/CD28/CD40 mAbs.

Results

Cocultivation of PBL or TIL with Autologous Tumor Cells. Six initial experiments were performed in which $CD8^+$ T lymphocytes purified from TIL were cultured with tumor cells, after which the supernatants were assayed for TNF or IFN-γ. In a representative experiment, $CD8^+$ TIL from a colon cancer patient (1C), first cultivated with 1C tumor cells for 15 days, were removed and added to either a fresh set of 1C cells or to tumor cells from a lung carcinoma patient (3L). A small amount of TNF (1.2 pg/ml) was detected when 1C lymphocytes were combined with the 1C but not with the 3L tumor, whereas TNF and IFN-γ (1.5 pg/ml) were produced when TIL from 3L were combined with 3L tumor cells but not when cultured alone. There was no evidence of lymphocyte proliferation. In subsequent experiments, TIL populations comprising monocytes, $CD4^+$ T cells, and B cells in addition to $CD8^+$ lymphocytes were combined with autologous tumor cells. Approximately 10 times higher levels of TNF (4.5–48 pg/ml) and up to 150 pg/ml of IFN then were detected in supernatants from cultures of eight of 13 patients. There was still no lymphocyte proliferation.

We then adapted a system in which mAb-conjugated magnetic beads are used to induce signals via various lymphocyte receptors (19, 20). PBL or TIL were combined with autologous tumor cells in the presence of beads conjugated with mAbs to CD3 and mAbs to CD28, alone or together with CD40. Similar groups were included with lymphocytes but without tumor cells. As controls,

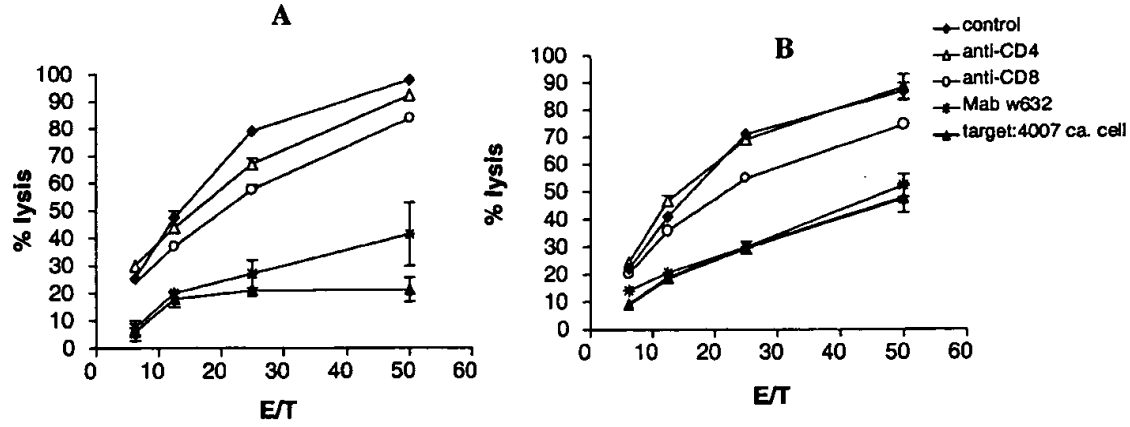

Fig. 3. Cr^{51} release data with PBL from 1C, tested on the indicated target cells, after activation on 1C cells by anti-CD3/CD28-conjugated beads (*A*) or anti-CD3/CD28/CD40-conjugated beads (*B*). Standard deviations were <10% of cpm. E/T, effector-to-target cell ratio.

lymphocytes, with or without tumor cells, were cultivated with control beads. After 3–5 days, the beads were removed and the lymphocytes were incubated separately over 2–21 days with 10 units/ml of IL-2.

Fig. 1 shows an experiment in which TIL from OV44 proliferated vigorously when exposed for 4 days to anti-CD3/CD28/CD40-conjugated beads. Lymphocytes cultivated in the absence of a CD3 signal did not proliferate and neither did lymphocytes cultured with anti-CD28 and/or CD40 beads (data not shown). Proliferation was greater when autologous tumor cells were initially present with the beads inducing signals via CD3 (Fig. 1*B*). Anti-CD3/CD28-conjugated beads induced proliferation similar to that with anti-CD3/CD28/CD40-conjugated beads (data not shown).

Fig. 2 shows an experiment in which PBL from patient 1C and various mAb-conjugated beads were cultivated for 5 days with either autologous tumor cells or allogeneic (4007) cells. The number of lymphocytes per culture was much higher when CD3/CD28 (Fig. 2*C*) or anti-CD3/CD28/CD40 (Fig. 2*D*) activated lymphocytes were combined with 1C tumor than with 4007 cells, a finding similar to that illustrated in Fig. 1. When, on the other hand, the beads did not provide any signal via CD3 (Fig. 2 *A* and *B*), the situation was the reverse and probably represented an immunological response to alloantigens expressed on the 4007 cells. FACS analysis showed that >90% of the activated lymphocytes expressed CD3 and ≈70% of them were $CD8^+$, with less than 5% expressing CD16 or CD56.

Most of the tumor cells were destroyed within 24–48 h after exposure to autologous lymphocytes in the presence of anti-CD3/CD28- or anti-CD3/CD28/CD40-conjugated beads, often leaving cultures entirely comprising cells with lymphocyte morphology. To study whether this destruction had immunological specificity, four experiments were performed in which serial dilution of PBL (10^6–10^5/sample) from cancer patients were combined with autologous tumor cells or with either tumor cells or fibroblasts from an allogeneic donor. In two experiments, there was ≈10 times more TNF in the culture supernatants in the presence of the autologous tumor, but there was no difference in the killing of cells from autologous or allogeneic tumors or of allogeneic fibroblasts. We conclude that tumor cell destruction seen after 24–72 h in the presence of lymphocyte activation was not immunologically specific, perhaps because large amounts of activated T lymphocytes and lymphokines obscured any specific components.

MHC-class I-restricted CTL were generated from lymphocytes activated by tumor cells plus anti-CD3/CD28 or anti-CD3/CD28/CD40 beads. Fig. 3 presents an experiment with PBL from 1C, which had been activated in the experiment shown in Fig. 2. After activation by tumor cells and mAb-conjugated beads, the beads were removed and the lymphocytes were expanded with 10 units IL-2/ml medium over 3 weeks in the absence of tumor cells and beads. PBL activated by 1C and anti-CD3/CD28 beads were strongly cytolytic to 1C cells, and lysis was inhibited by a mAb to CD8 and by anti-MHC class I framework mAb w6/32 (Fig. 2*A*). Allogeneic 4007 cells were killed by only 20% at an effector-to-target cell ratio of 50:1, as compared with 98% lysis of 1C cells (Fig. 2*A*). Fig. 2*B* demonstrates analogous data for PBL stimulated with anti-CD3/CD28/CD40 beads. Lysis of 4007 cells then was at the same low level as that of 1C in the presence of mAb w6/32. In contrast, PBL stimulated with anti-CD3/CD40 beads killed both 1C and 4007 cells, also in the presence of mAbs to CD8 or mAb w6/32 (data not shown). $CD8^+$ cells enriched from the cell population used in the experiment shown in Fig. 2*B* lysed 25% of 1C cells at an effector-to-target cell ratio of 1:20 as compared with 0% of cells from the 4007 line and 0% of cells from an allogeneic B cell line. In this experiment, lysis of 1C cells was 5% in the presence of mAb w6/32 and 5% with the anti-CD18 mAb 60.3, and it only decreased from 25% to 18% with a combination of mAbs to CD16 and-CD56. Lymphocytes activated by cocultivation with 4007 cells and any of the beads did not selectively lyse 1C or 4007 cells (data not shown). The CTL assays were repeated twice with similar results.

Large amounts of IFN-γ were detected in supernatants of cultures from lymphocytes activated via CD3 (Fig. 4). Fig. 4 also shows that the production of IFN-γ was higher when autologous tumor cells were present during the first 4–5 days of culture.

Table 1 presents six additional representative experiments show-

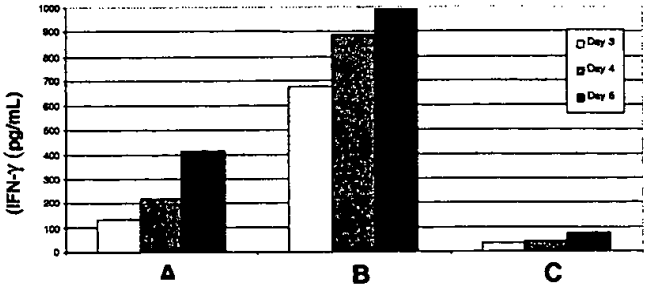

Fig. 4. IFN-γ produced by *in vitro* expanded PBL from 1HN. (*A*) No tumor, anti-CD3/CD28-conjugated beads. (*B*) Autologous tumor, anti-CD3/CD28-conjugated beads. (*C*) Autologous tumor, control beads (similar results without tumor).

Table 1. Proliferation (cell numbers × 10^6 per sample) and lymphokine production (pg/ml of TNF or IFN-γ) of freshly harvested PBL and TIL from patients with advanced cancer after culturing for 4–5 days ± autologous tumor cells in the presence of mAb-conjugated beads, followed by 2–4 days without beads (same time within each experiment)

mAb-conjugated beads	PBL								TIL				
	10V			80V		1HN*		480V*	30V			22C*	
	× 10^6	TNF	IFN-γ	× 10^6	TNF	× 10^6	TNF	× 10^6	× 10^6	TNF	IFN-γ	× 10^6	TNF
Control	1.1	30	479	4.2	12	1.3	3	0.4	1.2	0	271	1.4	5
Anti-CD3	11.3	2,440	5,560	15.3	1,550	4.8	2,080	NT	8.2	950	3,720	NT	NT
Anti-CD3/CD28	9.6	2,500	7,810	22.7	2,500	7.2	13,020	NT	7.0	2,100	4,730	5.8	1,660
Anti-CD3/CD40	7.8	2,500	4,480	18.6	>1,000	4.0	1,450	NT	5.4	660	3,060	3.6	1,540
Anti-CD3/CD28/CD40	NT	NT	NT	NT	NT	NT	NT	17.1	NT	NT	NT	6.5	2,060

Cultures were initiated with 10^6 PBL or TIL/sample. NT, not tested.
*Autologous tumor cells present together with the lymphocytes.

ing proliferation and lymphokine production by PBL or TIL, which were either tested upon harvest from the patients or after one round of *in vitro* activation with beads. Anti-CD3, anti-CD3/CD28, anti-CD3/CD40, and anti-CD3/CD28/CD40 beads strongly increased lymphocyte proliferation with no significant difference between them. In contrast, anti-CD28, anti-CD40, and anti-CD28/CD40 beads alone did not increase lymphocyte proliferation and lymphokine production over control beads (data not shown), indicating that signaling via CD3 was essential. Production of TNF and IFN-γ correlated well. It decreased to background levels when the lymphocytes were grown without tumor cells and beads for more than 3–5 days (data not shown). As in Figs. 1 and 4, CD3 signaling was required to induce vigorous lymphocyte proliferation and lymphokine production.

The density of CD antigen expression on lymphocyte populations was measured by FACS before and after 3- to 5-day cultivation with tumor cells and anti-CD3/CD28/CD40 beads, followed by an additional 3- to 7-day expansion without beads. To reflect changes in the density of CD receptor expression, the number of cells in each population whose brightness at least equaled the density at the chosen setting is reported (Table 2); unstimulated PBL from six healthy donors (30–65 years of age) were analyzed for comparison. Unstimulated PBL from the cancer patients had low levels of CD3, CD4 and CD28. Four of five patients also had low CD8 density, whereas the CD86 density was higher than among unstimulated PBL from the healthy donors. Culturing of PBL with control beads partially increased CD3 expression, but did not significantly increase CD28 expression. In contrast, culturing with anti-CD3/CD28/CD40 beads consistently restored the expression of CD3 and CD28 to normal levels, and it doubled the number of cells with high-density CD8 expression. Density of CD3 expression was studied with TIL from five patients. It was 2.9%, 40.2%, 96%, 42.8%, and 40.1%, respectively—i.e., it displayed more variation and was generally higher than for PBL. CD8 expression by TIL was higher than among PBL and increased from 61.4% to 87.3%. The corresponding figures for CD28 expression among TIL were 39.3% and 52.8%. Before cultivation, PBL and TIL from cancer patients comprised 10–20% $CD14^+$ cells (most likely monocytes). At 2 to 3 days after cultivation with anti-CD3/CD28 or anti-CD3/CD28/CD40 beads, 5–20% of the lymphoid cells were $CD83^+/CD3^-$; there was less than 1% of such cells with control beads or anti-CD28/CD40 beads.

To investigate whether an increased density of CD3 expression after *in vitro* activation of lymphocytes was due to the selective proliferation of cells with originally high CD3 expression, experiments were performed with TIL, 40.2% of which originally expressed CD3, which were labeled with the dye CFDA (25). After activation via anti-CD3/CD28/CD40 beads, CD3 expression increased to 95%. FACS analyses, using CFDA and phycoerythrin-labeled anti-CD3 as probes, showed that there was no selective proliferation of the subpopulation of PBL that originally had higher CD3 expression.

Effect of TGF-β1 in the Presence of Activation Signals via mAb-Conjugated Beads. Table 3 shows five representative experiments performed to investigate whether the inhibitory effect of TGF-β1 on lymphokine production and lymphocyte proliferation could be altered by coculture with beads inducing signals via CD3. With control beads, the TNF and IFN-γ levels were low, and these levels were further suppressed by TGF-β1. In contrast, with anti-CD3/CD28/CD40 beads these levels increased to levels often approaching those seen in the absence of TGF-β1. Likewise, when

Table 2. CD expression (mean ± SD) of PBL from six healthy adults and from cancer patients (5–8 patients per group), tested directly (unstimulated) or after culturing with mAb-conjugated beads for 4–5 days

Marker	Healthy donors, unstimulated	Cancer patients				
			Beads			
		Unstimulated	Control	Anti-CD3/CD28	Anti-CD3/CD40	Anti-CD3/CD28/CD40
CD3	72.3 ± 11	20 ± 24*	52 ± 32	92 ± 10†	96 ± 5†	94 ± 5†
CD4	45.4 ± 11	21 ± 20	37 ± 24	42 ± 23	29 ± 20	51 ± 18
CD8	21.7 ± 10	9 ± 7	18 ± 13	47 ± 26	70 ± 15†	45 ± 15†
CD28	62.1 ± 12	33 ± 17*	45 ± 32	79 ± 30	70 ± 36	93 ± 3†
CD56	2.2 ± 3	11 ± 12	25 ± 34	2 ± 4	3 ± 2	1.5 ± 2.4
CD80	0.1 ± 0	2 ± 2	4	1 ± 1	3 ± 5	5.6 ± 9
CD86	0.2 ± 0	34 ± 24*	10 ± 13	10 ± 14	16 ± 16	5 ± 3

Five to eight samples were tested per group of cancer patients.
*, $P < 0.01$ compared to unstimulated lymphocytes from healthy donors.
†, $P < 0.01$ compared to unstimulated lymphocytes from patients.

Table 3. Proliferation (cell numbers × 10^6 per sample) and lymphokine production (pg/ml of TNF or IFN-γ) by fresh or previously stimulated (*) PBL and TIL from cancer patients cultured for 4–5 days with tumor cells and mAb-conjugated beads ± (5 ng/ml) TGF-β1, followed by 2–3 days without beads or tumor cells but with TGF-β1 remaining

mAb-conjugated beads	TGF-β1 present	PBL								TIL		
		22C			480V		1HN*			480V		22C*
		× 10^6	TNF	IFN-γ	× 10^6	IFN-γ	× 10^6	TNF	IFN-γ	× 10^6	IFN-γ	× 10^6
Control	−	4.6	40	264	0.5	310	1.3	3	23	4.4	98	1.7
	+	2.8	0	19	0.5	19	0.4	5	48	2.8	27	1.4
Anti-CD3/CD28/CD40	−	19.7	2,900	>20,000	17.2	>10,000	7.2	11,680	24,050	8.1	9,810	6.5
	+	15.5	860	6,700	9.8	3,810	7.4	4,640	19,250	3.7	5,220	3.1

Cultures were initiated with 10^6 PBL or TIL/sample.

anti-CD3/CD28/CD40 beads were used, there was much less inhibitory effect of TGF-β1 on lymphocyte proliferation with no inhibition at all seen with patient 1HN. A relative resistance of T cell proliferation and lymphokine production was seen also when the TGF-β1 dose was increased to 20 ng/ml and when the concentration of lymphocytes was decreased to 10^5/sample (data not shown). Beads stimulating via CD28, CD40, alone or together, did not protect against TGF-β1 (data not shown).

Model Experiments with mAbs to V-β1 and V-β2 as Surrogate Antigens. Because stimulation via CD3 interferes with the induction of primary immune responses, experiments were performed in a model system to investigate the effect of CD3 stimulation on lymphocytes that had been activated by an antigen. As surrogate antigen, we used mAbs to V-β1 or V-β2, which allowed both the stimulation and recognition of $CD3^+$ T cells expressing specific T cell receptors. PBL from healthy donors were exposed to anti-V-β1 or anti-V-β2 mAbs either in the presence of control beads or beads conjugated with anti-CD3/CD28/CD40 mAbs.

The presence of anti-CD3/CD28/CD40 beads completely inhibited the induction of an immune response specific for anti-V-β1 or anti-V-β2 (data not shown). In contrast, as shown in two representative experiments (Table 4), signaling via these beads expanded the proliferation of V-β-specific T lymphocytes that had been previously activated. Lymphocytes primed by exposure to anti-V-β1 or anti-V-β2 proliferated, with retained specificity for the given anti-V-β, in response to the respective anti-V-β alone or in combination with anti-CD3/CD28/CD40 beads. Similarly, V-β specificity was retained when cells activated by anti-V-β were expanded with the anti-CD3/CD28/CD40 beads in the presence of the same anti-V-β mAb, a different one, or no such mAb. Stimulation via anti-V-β mAb together with activation signals via anti-CD3/CD28/CD40 beads led to higher production of TNF than achieved by antigen-specific stimulation or activation alone (Table 4, experiment 1). The presence of anti-CD3/CD28/CD40 beads protected against the inhibitory effect of TGF-β1 on lymphocyte proliferation and allowed the production of significant amounts of IFN-γ 2 days after removal of the beads from the lymphocytes and with TGF-β1 remaining in the culture medium (Table 4, experiment 2). With the anti-CD3/CD28/CD40 beads, >20,000 pg IFN-γ was detected in the culture medium whether or not TGF-β1 was present (data not shown). The number of T cells

Table 4. Secondary sensitization of PBL from two healthy donors in the presence of beads conjugated with anti-CD3/CD28/CD40 mAbs (or unconjugated beads, as controls) using anti-V-β1 or anti-V-β2 as surrogate antigens

Exp.	1st stimul. on anti-V-β	2nd stimul. on anti-V-β	Anti-CD3/CD28/CD40 beads	TGF-β	Lymphocytes (× 10^6)			Lymphokine pg/ml
					Total	V-β1	V-β2	
1	1	1	−	−	12.1	11.4	0.04	150
	1	2	−	−	2.5	1.9	0.05	NT
	1	None	−	−	1	0.79	0.01	1
	1	1	+	−	11.9	9.3	0.12	1,760
	1	2	+	−	14.0	11.3	0.28	NT
	1	None	+	−	15.7	12.9	0.16	880
	2	1	−	−	0.9	0.03	0.54	NT
	2	2	−	−	3.5	0.03	2.5	340
	2	None	−	−	0.5	0.005	0.32	2
	2	1	+	−	14.7	0.15	7.6	NT
	2	2	+	−	11.5	0.23	5.1	5,040
	2	None	+	−	13.9	0.14	7.2	1,960
2	1	1	−	−	12.1	8.0	0.1	1,220
	1	1	−	+	1.1	0.8	0.1	194
	1	1	+	−	14.9	6.5	0.1	1,546
	1	1	+	+	11.0	8.2	0.3	405
	1	2	−	−	4.4	3.7	0.3	47
	1	2	−	+	1.8	1.6	0.2	47
	1	2	+	−	19.1	12.1	0.2	536
	1	2	+	+	16.9	12.8	0.3	161

TGF-β1 (5 ng/ml) was added as indicated in exp. 2. Lymphocyte numbers (× 10^6) and lymphokine production (TNF in exp. 1 and IFN-γ in exp. 2) was measured 2 days after removal of the beads. NT, not tested.
*>90% of the lymphocytes are CD3-positive according to FACS analysis.

in groups exposed to anti-V-β1 and not receiving TGF-β1 was approximately the same whether or not anti-CD3/CD28/CD40 beads were present, whereas T cell proliferation sharply decreased when the group stimulated only via anti-V-β1 was exposed to TGF-β1. Consequently, the observed protection of CD3 engagement against inhibition by TGF-β1 was not an artifact caused by fewer molecules of TGF-β1 per T lymphocyte.

Discussion

Although initial experiments showed that PBL and TIL from patients with stage IV cancer secreted TNF and IFN-γ when cocultivated with autologous tumor cells, the lymphokine levels were extremely low (particularly in cultures lacking monocytes), and there was no lymphocyte proliferation. In contrast, lymphokine production was dramatically increased, and there was vigorous lymphocyte proliferation when we used a procedure (19, 20) in which beads conjugated with a mAb to CD3 in combination with mAbs to CD28 and/or CD40 were added to the cultures. Tumor-selective CTL, which were MHC class I-restricted and $CD8^+$, could be generated from lymphocytes that had been activated over 4–5 days by anti-CD3/CD28 or anti-CD3/CD28/CD40 beads in the presence of autologous tumor cells (and monocytes) and then expanded in the absence of tumor cells and beads. We conclude that stimulation of T lymphocytes via CD3 (and costimulatory signals) expands all T lymphocytes and facilitates the generation of CTL in the presence of autologous tumor cells and antigen-presenting cells in the cultures. The low proliferation and lymphokine production of unstimulated PBL harvested from cancer patients correlated with their low expression of CD3, CD28, CD4, and CD8. Exposure to anti-CD3/CD28 or anti-CD3/CD28/CD40 beads up-regulated lymphocyte expression of CD3 and CD28, as it increased their ability to proliferate and form lymphokines. Increased expression of CD3 was not the result of a preferential expansion of lymphocytes that originally had high density of CD3 expression.

Our data indicate that at least some patients with advanced cancers have T lymphocytes that can mount tumor-destructive immune reactions but are inhibited from doing so *in vivo*, and that signals mediated via CD3 in the presence of tumor antigens can activate these reactions. Because the view that polyclonal stimulation via CD3 can activate antitumor immunity challenges the current concept that such stimulation prevents or overrides recognition of antigen by the T cell receptor, experiments were performed in a model, using mAbs to V-β1 or V-β2 as surrogate antigens. These experiments demonstrated that signals mediated via CD3 dramatically expanded the proliferation of already primed T lymphocytes without loss of their V-β specificity whereas they prevented the *de novo* induction of a specific response. Furthermore, exposure of T lymphocytes to the specific anti-V-β together with anti-CD3/CD28/CD40 beads was optimal in inducing the production of TNF.

Tumor cells exposed to lymphocytes and stimulated via CD3 in combination with CD28 and/or CD40 were regularly destroyed within 24–48 h by a mechanism that had no detectable antigen specificity, although TNF production appeared to be greater in the presence of autologous tumor cells than allogeneic tumor cells or fibroblasts. Most likely, polyclonal stimulation of CD3 plus costimulation via CD28 and CD40 produced lymphokines that activated natural killer cells and monocytes and also (like TNF) had a direct toxic effect. We hypothesize that lymphocyte activation, accompanied by tumor cell killing, causes the release of antigen, which is taken up and processed by monocytes in the cultures that differentiate into dendritic cells and present epitopes for the selective expansion of tumor-reactive T cells. Therapeutic vaccines may be based on the same principle to activate and expand suppressed lymphocytes in tumor-bearing individuals and also may facilitate the generation of immune responses to subdominant epitopes. It is noteworthy that treatment of tumor-bearing mice with anti-CD3 mAb has been shown to have antitumor activity under certain circumstances (26).

The procedures we have used make possible the generation of $CD83^+$ dendritic cells and $CD3^+$ lymphocytes, which continue to expand over >10 weeks of *in vitro* culturing and should lend themselves to adoptive immunotherapy. This finding may be because costimulation via CD28 decreases the probability for lymphocytes to undergo apoptosis (27, 28), providing them with a long lifespan *in vitro* (19). Costimulated lymphocytes also have survived for a long time after transfer back to autologous patients (29) as opposed to lymphocytes expanded in the presence of high doses of IL-2.

Most of the patients died within a year of donating PBL or TIL, despite our evidence that their T cell repertoire at least in some cases included lymphocyte clones that could recognize the tumors as antigenically foreign. Most likely, the failure of these clones to expand *in vivo* and differentiate into effector cells was due to the production by the tumor and/or the host of molecules that down-regulated or terminated T cell reactivity. Many such molecules are known (9) with members of the TGF-β1 family being among the most powerful. The effects of TGF-β1 were thus investigated. It is encouraging that T cell stimulation via CD3 in combination with CD28 alone or together with CD40 protected against ≈50% of its inhibitory effect on lymphocyte proliferation and production of TNF and IFN-γ, even when the TGF-β1 was used at saturation levels of 20 ng/ml in the cultures. Likewise, T cell stimulation via anti-CD3/CD28/CD40 beads protected against the inhibitory effect of TGF-β1 in the anti-V-β model.

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Clinical-Scale Selection of Anti-CD3/CD28–Activated T Cells After Transduction with a Retroviral Vector Expressing Herpes Simplex Virus Thymidine Kinase and Truncated Nerve Growth Factor Receptor

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ABSTRACT

Activation of T cells is necessary for efficient retroviral-mediated gene transfer. In addition, if the population of infused cells is to be limited to transduced cells, a means of positive selection is required. We describe a clinical scale procedure for activation of donor T cells with anti-CD3/CD28 beads followed by transduction with a retroviral construct expressing the herpes simplex virus thymidine kinase (HSV-tk) and human nerve growth factor receptor (NGFR). Optimization of transduction parameters was performed, testing the timing of transduction, centrifugation, and the use of serum. In large-scale experiments, 3–5 × 10^8 peripheral blood mononuclear cells (PBMC) were activated with anti-CD3/CD28 beads and expanded to day 13. Transduction was accomplished using MFG-TKiNG supernatant produced from the PG13 packaging line 48 hr after T-cell activation. The mean transduction frequency was 37.5% based on NGFR expression, and the mean expansion observed was 42.6-fold (mean final cell number 1.85 × 10^{10}). A comparison of the ability of the Baxter Isolex 300i and the Miltenyi CliniMACS to perform purification of NGFR+ cells suggests that greater purity can be achieved with the CliniMACS device (67.4% vs. 97.7%), while the yield of transduced cells appears higher with the Isolex 300i (41.3% vs. 23.5%). We conclude that a strategy based on activation of human T cells with anti-CD3/CD28 beads can result in sufficient transduction, expansion, and purification based on NGFR expression for clinical trials.

OVERVIEW SUMMARY

The potential use of T cells engineered with negative selectable elements such as the herpes simplex virus thymidine kinase gene may prove advantageous by achieving additional efficacy and safety associated with allogeneic transplantation. If retroviral-mediated gene transfer is to be used, the development of clinical scale methodology for the activation, transduction, expansion, and selection of sufficient T cells is of great importance, and is the focus of these investigations. Because of concerns regarding the possible induction of apoptosis via activation through the T-cell receptor alone and/or interleukin-2, we chose to test magnetic beads with anti-CD3 as well as anti-CD28 antibodies to obtain activation to allow efficient transduction. The roles of retroviral packaging lines, centrifugation, and serum were evaluated. In addition, the utilization of the nerve growth factor cell surface antigen and magnetic bead technology as a positive selection strategy was tested to compare magnetic bead devices to determine the relative purity and yield that can be achieved in preclinical studies.

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INTRODUCTION

Allogeneic hematopoietic cell transplantation (HCT) is an important therapeutic modality for malignancies, marrow failure, metabolic disorders, and immunodeficiencies. Unfortunately, the morbidity and mortality associated with graft versus host disease (GVHD) remains high (Nademanee *et al.*, 1995; Davies *et al.*, 1996). The cells primarily responsible for GVHD are donor T cells, and the incidence and severity of GVHD can be decreased with T-cell depletion (TCD) of the graft. However, the removal of the T-cell population may be associated with an increased risk of graft failure, and may also adversely affect immune reconstitution (Martin *et al.*, 1985; Marmont *et al.*, 1991; Davison *et al.*, 2000). In addition, the beneficial immunologic surveillance provided by donor T cells decreases the relapse rate of recipients undergoing transplantation for leukemia, and this graft versus leukemia (GVL) effect may be diminished by T-cell depletion (Gale and Horowitz, 1990; Horowitz *et al.*, 1990; Enright *et al.*, 1996). Therefore, strategies that allow the administration of donor T cells to facilitate engraftment and GVL while providing an additional means of controlling GVHD may prove beneficial.

One approach developed to address these issues is the genetic engineering of donor T cells with a gene providing the potential for negative selection (Bonini *et al.*, 1997; Tiberghien *et al.*, 1997). This "suicide gene" approach allows the eradication of the engineered cell population with a prodrug that is relatively nontoxic to cells not expressing the gene. Thus, this approach could allow the inclusion of T cells with their positive effects on engraftment, GVL, and immunologic function while providing an additional measure of protection from severe GVHD. There have been reports suggesting that this is feasible in murine models (Cohen *et al.*, 1997; Contassot *et al.*, 2000), and may prove advantageous in a clinical setting (Bonini *et al.*, 1997; Tiberghien, 2001; Tiberghien *et al.*, 2001). The successful implementation of this procedure requires the resolution of several obstacles. If retrovirus-mediated gene transfer is used, the target cells must be proliferating for effective proviral integration (Miller *et al.*, 1990). Second, because it is unlikely that all T cells will be transduced, some means of positive selection will be required to ensure that the T cells to be infused contain the capacity to provide negative selection. In addition, the T cells that have undergone activation, transduction, and selection may prove phenotypically or immunologically distinct from unmanipulated T cells ordinarily infused with an marrow allogeneic graft. Finally, the ability to eliminate the transduced cell population at a concentration of prodrug that can be achieved in the serum of patients undergoing HCT is necessary.

We report the results of our clinical-scale cell production, transduction, and selection studies using magnetic beads with antibodies to both CD3 and CD28 to provide the activation signals to T cells to achieve high-efficiency transduction. Signaling through both the T-cell receptor and the costimulatory molecule CD28 has been documented to initiate proliferation of T cells (Levine *et al.*, 1998; Garlie *et al.*, 1999; Shibuya *et al.*, 2000), and may decrease the development of anergy or apoptosis associated with stimulation through the T-cell receptor alone (Wolf *et al.*, 1994; Noel *et al.*, 1996; Muller *et al.*, 1999). The use of anti-CD3 antibodies and interleukin-2 (IL-2) has been shown to generate large numbers of T cells (Anderson *et al.*, 1988; Ochoa *et al.*, 1989), and has been used to provide transduced T cells for clinical use (Tiberghien *et al.*, 2001). However, there is concern that the use of antibodies capable of cross-linking the T-cell receptor alone may result in eventual apoptosis of the activated cells, which may be prevented with stimulation via the CD28 costimulatory pathway, possibly by modifying expression of bcl-2 and bcl-x (Noel *et al.*, 1996; Radvanyi *et al.*, 1996; Muller *et al.*, 1999). We therefore chose to test the potential to achieve transduction of human T cells utilizing anti-CD3/CD28 antibodies bound to magnetic beads (Levine *et al.*, 1995, 1996). We have utilized a vector expressing the herpes simplex virus thymidine kinase (HSV-tk) gene to provide the capacity for negative selection. Transduced cells were identified and purified on the basis of the cell surface expression of the truncated human nerve growth factor receptor (NGFR). The Baxter Isolex 300i (Nexell Therapeutics Inc., Irvine, CA) and Miltenyi CliniMACS (AmCell Corp., Burlingame, CA) clinical cell separation devices were compared to determine purity and yield of NGFR+ human T cells. We determined that peripheral blood mononuclear cells (PBMC) activated with anti-CD3/CD28 beads and IL-2 achieved a sustained proliferative response. In association with a transduction protocol using a vector packaged to make use of gibbon ape leukemia virus (GALV) envelope and centrifugation, transduction frequencies of 40%–60% were achievable. In large-scale transduction experiments, the Baxter Isolex 300i appeared to provide a greater yield of NGFR+ cells, while a higher degree of purity was achieved with the Miltenyi CliniMACS device. We believe this is the first report of a clinical-scale protocol in which anti-CD3/CD28 beads are used to achieve T-cell transduction and selection on the basis of expression of a cell surface antigen such as NGFR.

MATERIALS AND METHODS

Mammalian cell culture

NIH 3T3 cells, PA317 and PG13 cells were maintained in Dulbeccos's Modified Essential Medium (DMEM) supplemented with 10% newborn calf serum (Sigma, St. Louis, MO) and penicillin/streptomycin (GIBCO BRL, Rockville, MD). Retroviral supernatants were generated in small scale from confluent retroviral producing lines in 75-cm^2 flasks (Corning, Corning, NY) at 32°C over 16–24 hr. Large-scale virus production was performed using a NUNC cell factory apparatus (Cat. #164327; NUNC, Naperville, IL) by placing fresh medium every 24 hr on the confluent producer line maintained at 32°C as long as the cells remained adherent. Supernatants generated from viral producer lines were filtered using 0.22-μm filters (Cat. #357111, Becton Dickinson Labware, Franklin Lakes, NJ) to remove any residual producer cells. In proliferation assays 10^5 cells were placed in a total of 200 μl of medium in 96-well plates, and were pulsed for 16 hr with 1 μCu of [^{3}H]thymidine (Perkin Elmer Life Sciences Inc., Boston, MA) prior to cell harvesting and determinations of thymidine incorporation (cell harvester, matrix 9600 reader; Packard Instruments, Downer's Grove, IL).

Blood samples were obtained from volunteer donors in accordance with University of Minnesota Institutional Review Board (IRB)-approved protocols using heparin-containing 10-ml

tubes; in situations in which a unit of whole blood was used, 5000 units of heparin were added to the bag containing the specimen prior to processing. PBMC were obtained using histopaque (Cat. #1077-1, Sigma) separation. The cells were maintained in X-VIVO 15 (Biowhittaker; Walkersville, MD) with penicillin/streptomycin and either 10% fetal bovine serum (FBS) or human frozen plasma (FP) purchased from the Red Cross (St. Paul, MN). Prior to addition to X-VIVO 15, the FP was thawed, maintained at 55°–57°C for 1 hr and centrifuged at 3000 rpm (2510*g*) for 10 min at room temperature. Using a plasma extractor, the plasma was expressed into 300-ml transfer bags, allowing any clots to remain in the original bag. Activation of PBMC was achieved with anti-CD3/CD28 beads added to the cells in culture on day 0 at a 3:1 ratio to the number of T cells (Levine *et al.*, 1998; Garlie *et al.*, 1999). IL-2 (Chiron Corp., St. Louis, MO) was used in individual experiments as described.

Monoclonal antibodies and flow cytometric analysis

The anti-NGFR hybridoma 20.4 (murine IgG1) was obtained from ATCC (HB 8737, 200-3-G6-4; clone 20.4) and antibody produced for our laboratory by Taconic BioServices (Germantown, NY). Depending on the experiment, either unconjugated or biotinylated antibody was used for detection or selection of NGFR+ cells. Additional antibodies used for phenotypic analysis including anti-CD3 were obtained from Becton Dickinson; flow cytometric analysis was performed using a Becton Dickinson FACSCaliber.

Construction of the MFG-TKiNG vector

The MFG-TKiNG retroviral construct was designed to express a truncated form of NGFR and HSV-tk using the MFG strategy, which has been shown to provide high-efficiency gene transfer and expression (Dranoff *et al.*, 1993; Jaffee *et al.*, 1993). The GCsamE75t vector consists of the Moloney murine leukemia virus long terminal repeats (LTR), retroviral splice donor/acceptance sites, a polylinker including the *Not*I and *Xho*I sites, an internal ribosomal entry site (IRES) and the NGFR cDNA. The HSV-tk gene was isolated from plasmid pHSV-106 (Bethesda Research Laboratories Inc., Gaithersburg, MD) and was ligated into the *Not*I/*Xho*I polylinker in frame with the NGFR gene using the encephalomyocarditis IRES (Zitvogel *et al.*, 1994; Gallardo *et al.*, 1997) to express both the NGFR and HSV-tk genes from a single bicistronic message, optimizing the potential that the HSV-tk gene will be expressed in cells selected on the basis of NGFR (Fig. 1). The construct was cotransfected into the PA317 amphotropic line (Miller and Buttimore, 1986) with the pFR400 plasmid containing the Arg22 dihydrofolate reductase (DHFR) gene and colonies selected in 1.0 μM methotrexate (Simonsen and Levinson, 1983). Clones were screened for their production of HAT resistant colonies on NIH 3T3 tk$^-$ cells as previously described (Vinh and McIvor, 1993). The highest titer observed was 0.8×10^6 colony-forming units per milliliter (cfu/ml) of supernatant. Supernatants from PA317 clones were used to transduce the PG13 line (Bauer *et al.*, 1995; Bunnell *et al.*, 1995), and clones selected on the basis of NIH 3T3 tk$^-$ expression using flow cytometry to sort the NIH 3T3 tk$^-$ population with the anti-NGFR antibody 20.4. Because the GALV receptor is not expressed on murine cells, the titer of these clones could not be determined on NIH 3T3 tk$^-$ cells; therefore, the relative rates of transduction were determined in donor-derived human T cells.

T-cell transduction assay

In the small-scale analysis used to optimize transduction conditions, transduction of human T cells was performed 48 hr after activation of T cells, unless otherwise noted. Ficoll separation was used to obtain PBMC, which were activated with anti-CD3/CD28 beads at a ratio of 3 beads per cell in 1000 units of IL-2 per milliliter in X-VIVO 15 medium with 10% FBS, unless otherwise stated. After 48 hr, 2×10^5 cells were placed in 15-ml polypropylene tubes (Corning) in 0.4 ml of X-VIVO 15 and 1.2 ml of retroviral supernatant; protamine (American Pharmaceutical Partners, Inc., Los Angeles, CA) was added to achieve a final concentration of 8 μg/ml. Unless otherwise indicated, the cells were centrifuged at 4000*g* at 32°C for 1 hr in a JS-30 rotor in a J-6B centrifuge (Beckman Coulter, Inc., Fullerton, CA), and incubated for 16 hr at 37°C in 5% CO_2. The cells were washed and placed in fresh culture medium, and after 48 hours, flow cytometry was performed with 100 ng of the unconjugated 20.4 anti-NGFR antibody and 10 μl of a secondary anti-murine immunoglobulin G (IgG) antibody conjugated to phycoerythrin (PE) (Sigma). The cells were also stained with an anti-CD3 antibody conjugated to fluorescein isothiocyanate (FITC). In other experiments, modifications in the transduction procedure were made as noted in the figures.

Clinical-scale experiments

To obtain sufficient cells for large-scale testing, 1 unit of whole blood was procured from volunteer donors in accordance with an IRB-approved protocol, and Ficoll separation performed. On day 0 a total of $3–5 \times 10^8$ PBMC were activated with anti-CD3/CD28 beads at a 3:1 ratio, and the cells were cultured in X-VIVO supplemented with 10% FP and penicillin/streptomycin to achieve a final concentration of 0.5×10^6 cells/ml in 1000 units of IL-2 per milliliter in a 3-liter Lifecell bag (Dynal Biotech Inc., Lake Success, NY). On day 2, 1.5 liters of retroviral supernatant (PG13-derived) and protamine

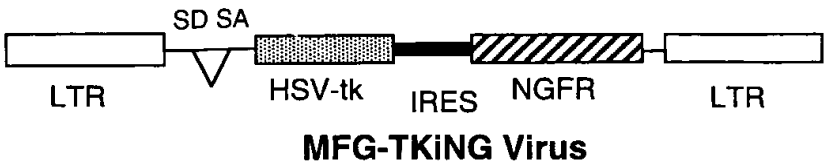

FIG. 1. MFG-TKiNG retrovirus. The herpes simplex virus thymidine kinase gene (HSV-tk) and the truncated nerve growth factor receptor (NGFR) are coexpressed using the internal ribosome entry site (IRES) from the encephalomyocarditis virus. Splice donor (SD) and splice acceptor (SA) sites are designated.

(final concentration, 8 μg/ml) were added to the cells. The product was separated into six 600-ml transfer bags (Terumo Medical Corporation, Somerset, NJ) and centrifuged at 3710 rpm (4000*g*) for 1 hr at 32°C in a Sorvall RC-3B centrifuge and then carefully removed and placed upright in a 37°C 5% CO_2 incubator. On day 3 the cells were washed and placed in fresh medium in a concentration of 0.5×10^6 cells/ml. Fresh medium was added every 2–3 days to keep the cell number less than 2×10^6 cells/ml until day 13, when the anti-CD3/CD28 beads were removed using the secondary magnet of the Baxter Isolex 300i device (Nexell Therapeutics Inc.). A sample of cells was obtained to determine the proportion expressing NGFR, and the remainder was subjected to separation of the NGFR+ fraction using the Isolex 300i or the CliniMACS device. To perform the isolations, 1.0 ml of the anti-NGFR monoclonal antibody 20.4 (2.4 mg) was added to the cells. In the case of the experiments using the Isolex 300i, an unconjugated 20.4 antibody preparation was used in association with the sheep anti-murine antibody/bead reagent currently being used in clinical trials according to manufacturers' specifications. For selections using the CliniMACS device, 1.0 ml of a biotinylated 20.4 antibody was used with streptavidin beads (Miltenyi Biotech Inc.) according to specifications, using the tubing sets and buffers designed for the CliniMACS.

RESULTS

Activation and timing of transduction in donor-derived T cells

To achieve high-efficiency retrovirus-mediated gene transfer it is critical to induce the target cell population to undergo mitosis to facilitate integration (Miller *et al.*, 1990; Stevenson *et al.*, 1990). In these studies, anti-CD3/CD28 beads were utilized to activate T cells by cross-linking the T-cell receptor and costimulatory pathways simultaneously. We first sought to determine the rate at which proliferation was achieved in PBMC after exposure to the anti-CD3/CD28 beads, comparing thymidine incorporation to cells induced with IL-2 alone or both IL-2 and beads. Active proliferation was observed in cells pulsed 24-hr postactivation with the beads, and was not affected by the presence or absence of exogenous IL-2 (100 U/ml in this experiment). Thymidine incorporation continued over the 96 hr of the assay in cells in the presence of anti-CD3/CD28 beads, whereas little proliferation was documented in the absence of the beads, despite the addition of IL-2 to the culture (Fig. 2).

Centrifugation as a means of enhancing T-cell transduction

The role of centrifugation as a strategy for increasing the efficacy of retroviral mediated gene transfer was tested to determine effects on transduction frequency and overall viability. PBMC activated with anti-CD3/CD28 beads were transduced 48 hr after activation, using protamine (8 μg/ml). In this experiment, two donors were used, and testing at each point performed in triplicate for each donor. The reported data is pooled from both donors ($n = 6$), as a similar trend was observed in both donors (Fig. 3). We observed a continuous increase in transduction frequency to 4000*g* with minimal effect in the viability of the transduced cells. We chose 4000*g* for subsequent experiments and did not continue to increase the *g* force, because there were concerns regarding the integrity of the bags with additional centrifugation.

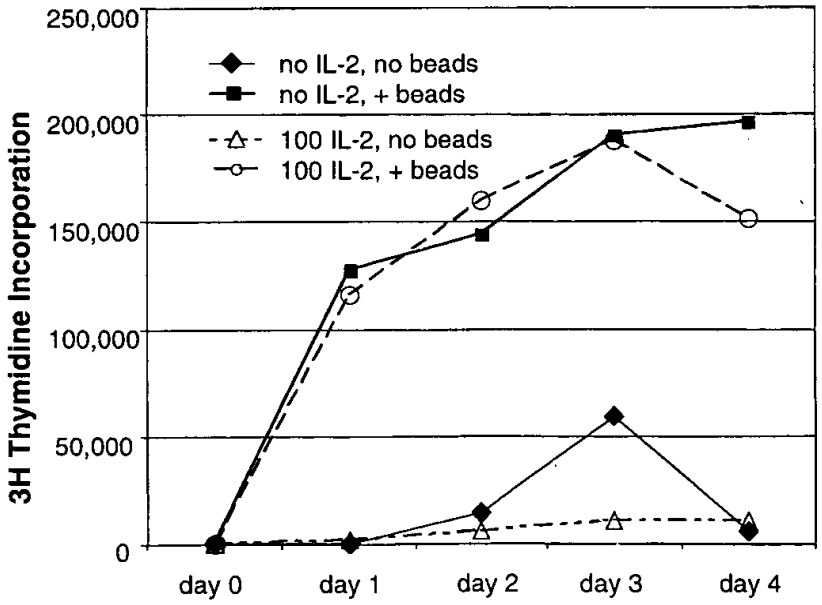

FIG. 2. Proliferation of human T cells with anti-CD3/CD28 beads. Donor-derived peripheral blood mononuclear cells (PBMC) were activated with anti-CD3/CD28 beads at a ratio of 3 beads per cell in the presence or absence of 100 units of IL-2 per milliliter. The day that PBMC were isolated, cells in each group were pulsed with [^{3}H]thymidine (day 0). Cells in identical groups were pulsed at 24, 48, 72 and 96 hr in culture, corresponding to day 1 through 4.

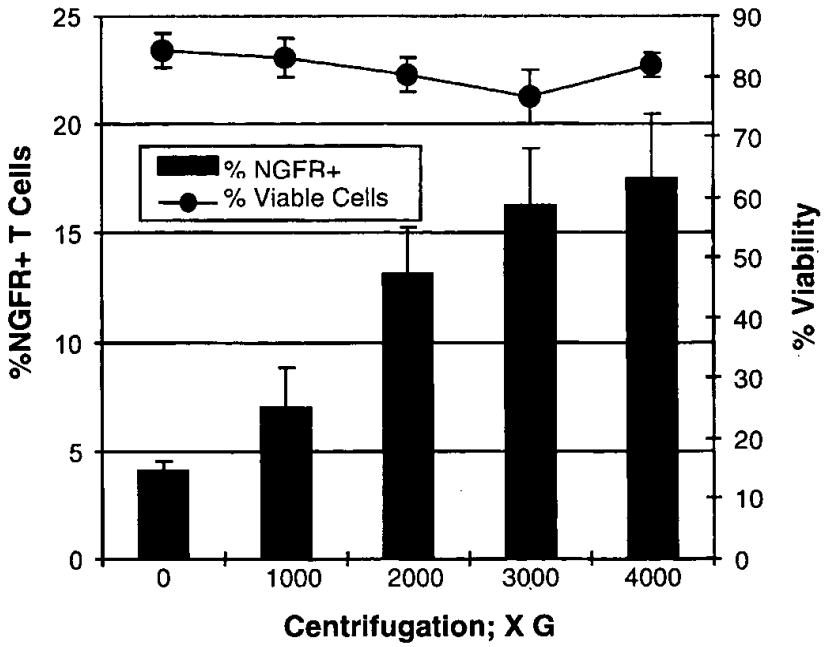

FIG. 3. Effect of centrifugation on transduction frequency and T cell viability. Peripheral blood mononuclear cells (PBMC) activated with anti-CD3/CD28 beads were transduced 48 hr postactivation in triplicate in X-VIVO 15 medium with 1000 U/ml IL-2. In each tube 2×10^5 cells in 400 μl of medium were transduced using 1.2 ml of MFG-TKiNG supernatant in 8 μg/ml protamine using centrifugation (0–4000*g*) for 1 hour at 32°C. Supernatant derived from PG13 supernatant was used for transduction. Trypan blue was used to determine viability while anti-nerve growth frequency receptor (NGFR) antibody was used to evaluate the frequency of transduction.

Transduction efficacy using PA317 and PG13 packaged vector

To achieve maximal transduction efficacy, we sought to determine if packaging lines containing the amphotropic murine leukemia virus (A-MuLV) or GALV envelope gene was most advantageous in achieving gene transfer and expression in anti-CD3/CD28 activated T cells. The MFG-TKiNG plasmid was initially transfected into the A-MuLV line PA317, and supernatant used to transduce PG13 cells, as described above. Donor-derived PBMC were exposed to retroviral supernatants 48 hr after activation in the presence of IL-2 (1000 U/ml) in triplicate. The advantage of using supernatant generated from the GALV line (PG13) was straightforward and highly significant (Fig. 4).

Timing of transduction after activation

The next goal was to establish the optimal timing of transduction after activation with the anti-CD3/CD28 beads; PBMC were activated and transduced in replicates of 3 using MFG-TKiNG supernatant 24, 48 or 72 hr after activation. In this experiment, 1.2 ml of retroviral supernatant (PG13-derived) was used to transduce 2×10^5 activated cells in 0.4 ml of medium; the proportion of cells expressing NGFR was determined by flow cytometry after transduction. This documents that efficient transduction (median, 58%) can be achieved with a single exposure of retroviral supernatant when transduction is performed 48 hr after initial activation (Fig. 5).

Role of serum products in the transduction of human T cells activated with anti-CD3/CD28 beads

The use of serum-free medium such as X-VIVO 15 may be advantageous in supporting the proliferation of T cells without a requirement for human or bovine serum. We therefore determined the effect of serum products such as FBS or FP on the transduction of T cells. From the initial placement of PBMC in X-VIVO 15 medium, no serum, 10% FBS, or 10% FP was provided within the medium, which continued throughout the culture period including the transduction procedure. Retroviral supernatant used for transduction in each group was from a single batch of supernatant produced in DMEM medium and 10% FBS. We observed that the addition of FBS to the medium increases transduction efficiency when compared to cells maintained in medium without serum, while the use of FP provided a significantly increased transduction frequency when compared to FBS (Fig. 6).

Large-scale expansion and selection of transduced T cells based on NGFR expression

For these experiments, volunteer donors provided 1 unit of whole blood from which PBMC were obtained. The mean number of PBMC was 5.8×10^8 cells (±2.4 standard deviations [SD]). For each experiment 5×10^8 PBMC were activated with anti-CD3/CD28 beads, except in two cases when less than 5×10^8 cells were obtained (2.9 and 3.0×10^8 cells) and expanded in the presence of anti-CD3/CD28 beads and IL-2 to day 13.

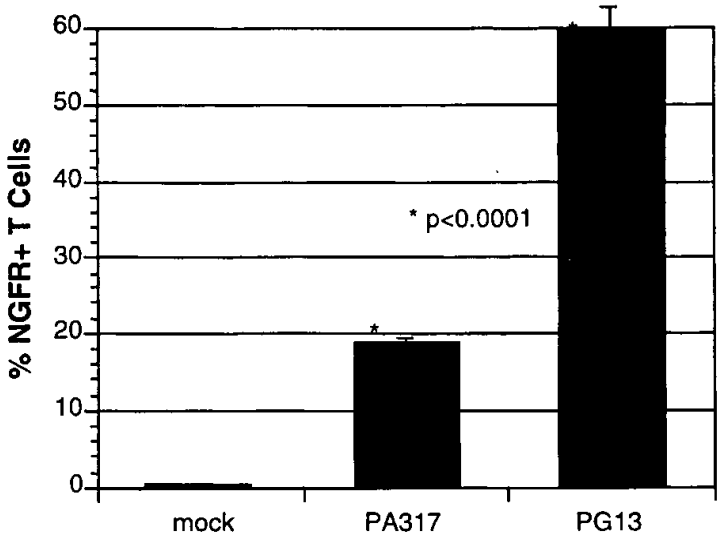

FIG. 4. Transduction of peripheral blood mononuclear cells (PBMC) with PA317 and PG13 supernatant. MFG-TKiNG supernatant derived from the PA317 and PG13 lines were used to transduce donor-derived T cells in triplicate 48 hr after activation by anti-CD3/CD28 beads in X-VIVO 15 medium with 1000 U/ml of IL-2 using protamine (8 μg/ml) and centrifugation (4000*g* for 1 hr at 32°C). Transduction frequency was determined by flow cytometric analysis of nerve growth factor receptor (NGFR) expression 72 hr posttransduction.

The mean number of viable cells present after activation, transduction, and expansion was 1.85×10^{10} ($\pm 0.93 \times 10^{10}$) cells, with a mean expansion of 42.6-fold. The cells were transduced with the MFG-TKiNG virus on day 2, and the mean transduction frequency was 37.5% ± 26.8% ($n = 5$). Because the purpose of this strategy is to ensure that infused T cells can be eradicated *in vivo* if severe GVHD is observed, a means of achieving purity of successfully transduced T cells on the basis of NGFR+ is of great importance. We therefore sought to compare the two clinical scale devices available for selection of cell surface antigens using magnetic beads. The Baxter Isolex 300i and the Miltenyi CliniMACS device have both been de-

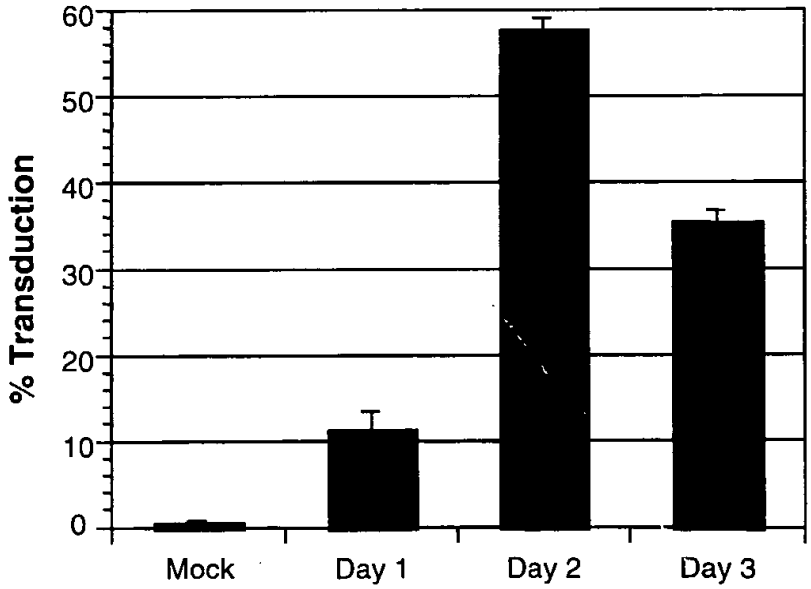

FIG. 5. Transduction frequency based on timing after activation. Donor-derived peripheral blood mononuclear cells (PBMC) were activated with anti-CD3/CD28 beads in X-VIVO 15 medium with 1000 U/ml IL-2, and 24 hr later 2×10^5 cells in 400 μl were transduced using MFG-TKiNG supernatant (1.2 ml) in the presence of 8 μg/ml of protamine (day 1). Transduction was performed using centrifugation (4000*g* for 1 hr at 32°C) and transduction allowed to proceed for 16 hr at 37°C. On day 2 (48-hr postactivation) the day 1 transduced population was washed and resuspended in fresh medium, while additional cells (day 2) underwent transduction using an identical procedure; similarly, transduction was performed in the final group (day 3) 72 hr after initial activation. The proportion of cells expressing nerve growth factor receptor (NGFR) was determined 48 hr after transduction of the third group.

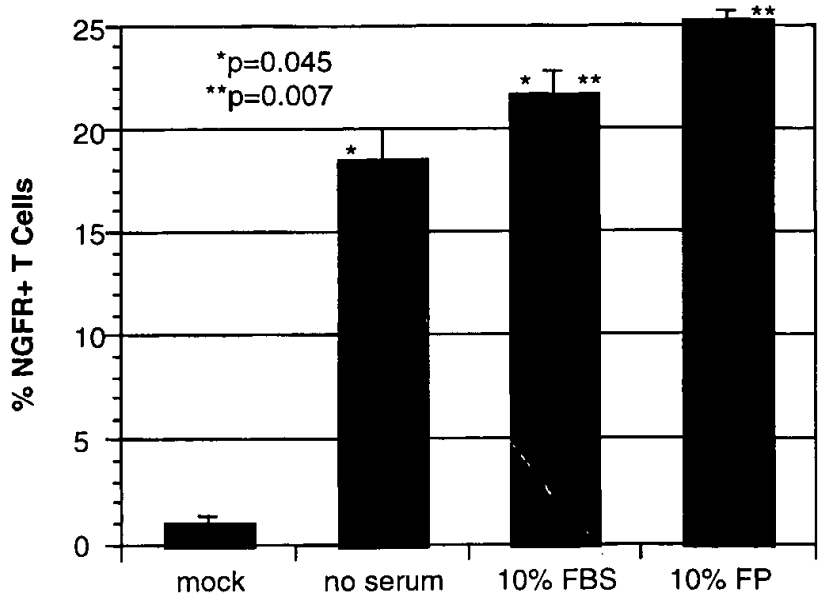

FIG. 6. Effect of serum and frozen plasma (FP) on transduction frequency. Peripheral blood mononuclear cells (PBMC) activated as described previously were cultured in X-VIVO 15 medium with 1000 U/ml of IL-2 alone (no serum) or with 10% fetal bovine serum (FBS) or human FP throughout the experiment. Transduction was performed 48 hr after activation using centrifugation and protamine as previously described.

veloped to perform positive selection of hematopoietic cells for clinical applications. In these experiments, the cells were divided after transduction and expansion and separations performed on both devices as described above. The findings suggest that greater purity may be obtained with the CliniMACS device (mean proportion of NGFR+ cells 97.7% vs. 67.4%, respectively; $p = 0.051$), while a greater recovery of NGFR+ cells was observed with the Isolex 300i (mean yield 41.3% vs. 23.5%), although statistical significance was not achieved (Fig. 7).

DISCUSSION

In this report we describe a preclinical protocol to generate large numbers of transduced human T cells after activation with anti-CD3/CD28 beads, using a retrovirus designed to express HSV-tk. A high degree of purity of the transduced cell population is achieved on the basis of NGFR using magnetic beads and clinical scale devices. To develop procedures designed to generate sufficient numbers of cells for clinical trials, activation of T cells is of importance to achieve transduction. We chose to investigate the use of anti-CD3/CD28 beads to activate T cells in experiments designed to test the feasibility of this approach to provide a protocol sufficient to generate cells for clinical purposes. The use of this technique has been reported as resulting in a population of T cells that can be continuously stimulated to actively proliferate (Levine *et al.*, 1996; Garlie *et al.*, 1999). Our data confirm that highly efficient gene transfer can be achieved in human T cells with a single exposure to the MFG-TKiNG retrovirus. These findings are similar to those of other investigators using immobilized anti-CD3 and anti-CD28 antibodies (Pollok *et al.*, 1999; Koehne *et al.*, 2000). In addition, Quinn et al. described the potential to transduce human T cells after activation with these antibodies immobilized on beads (Quinn *et al.*, 1998), although not in numbers sufficient for clinical use and without the capacity to perform positive selection on the basis of the expression of cell surface antigens. The described protocol provides efficient retroviral-mediated gene transfer, expansion, and selection using clinical scale magnetic bead devices.

The choice of packaging cell line has important implications for the transduction of human hematopoietic cells. The PA317 line, expressing the amphotropic murine leukemia virus (A-MuLV) envelope, has been well-tested clinically and provides the advantage of facilitating titering of virus using murine thymidine kinase negative (tk-) cell lines (Tabin *et al.*, 1982; Miller *et al.*, 1985). In contrast, the PG13 line was established to express the GALV envelope, and supernatants produced using this line have been shown to provide enhanced gene transfer in human hematopoietic cells (Bauer *et al.*, 1995; Bunnell *et al.*, 1995). However, because virus produced using PG13 cells is unable to transduce murine cells, the use of other means to quantitate the MFG-TKiNG virus is necessary. Comparison of the PA317 derived supernatant (titer of 0.8×10^6 cfu/ml based on determinations using the tk-3T3 cells) and PG13 supernatants in donor-derived T cells documents the increase in transduction frequency achieved with the latter.

Centrifugation has been used to enhance the physical association of retrovirus and target cells, and the effects on transduction have been shown to increase with the *g* force utilized as well as the duration of centrifugation (Bahnson *et al.*, 1995). We were interested in determining the effect of centrifugation on transduction frequency and T-cell viability. An additional issue in large-scale transduction procedures in the capacity for tissue culture bags to withstand the centrifugation procedure. Our data support previous observations that increases in the *g* forces associated with centrifugation increases the transduction frequency, while little decrease in viability was observed to 4000*g*. The 600-ml transfer bags were not susceptible to failure as tested.

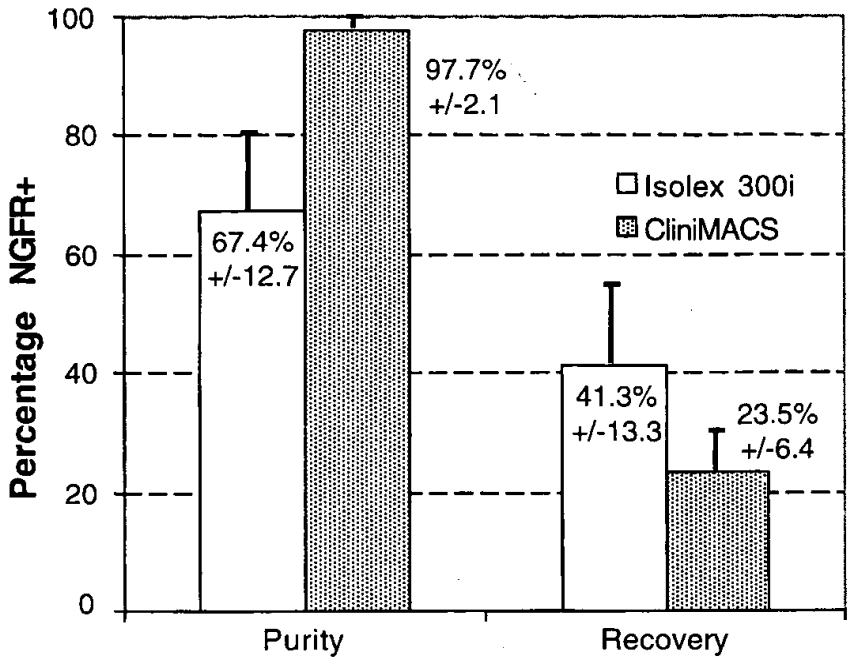

FIG. 7. Comparison of selection of nerve growth factor receptor-positive (NGFR+) cells using the CliniMACS and Isolex 300i Devices. Large-scale transduction experiments were performed using a mononuclear cell population derived from a unit of whole blood after separation using Ficoll. The cells were activated with anti-CD3/CD28 beads and transduced 48 hr later in X-VIVO 15 with 10% frozen plasma (FP) and 1000 U/ml of IL-2. Expansion continued until day 13, at which time the cells were divided and selected on the basis of NGFR expression using the Baxter Isolex 300i or Miltenyi CliniMACS device. The expression of NGFR was determined by flow cytometric analysis prior to and after selection. The total yield was determined by calculating the number of NGFR+ cells in a given cell fraction, and comparing this to the number of cells and the proportion that were NGFR+ after selection.

Modification of T cells for the purposes of both purification of transduced cells (positive selection) as well as the ability to eradicate the cells *in vivo* (negative selection) requires expression of two genes from the same virus. The retrovirus constructed for this purpose (MFG-TKiNG) has been designed to express the truncated NGFR gene as well as HSV-tk using the MFG strategy, which has been shown to provide efficient gene transfer and expression in a number of target cells (Dranoff *et al.*, 1993; Jaffee *et al.*, 1993). Our laboratory has previously used MFG virus successfully in human natural killer (NK) cells (Miller *et al.*, 1997). The positive selectable element utilized in the MFG-TKiNG viral construct is the NGFR gene, which has been shown to be expressed well on human T cells (Mavilio *et al.*, 1994; Valtieri *et al.*, 1994). The NGFR molecule also has the potential advantage of being less immunogenic, because it has been documented that immunologic responses can be directed against antibiotic resistance genes (Riddell *et al.*, 1996; Verzeletti *et al.*, 1998). The use of the IRES in the vector design was designed to provide an additional measure of safety, providing expression of both genes on the same bicistronic message while selecting on the basis of the second gene. We observed significant variation in the rates of transduction in donor derived human T cells from experiment to experiment, likely caused by the titer of supernatant as well as differences between donors and experimental variation.

Culture conditions may prove of significant importance in optimizing the expansion, viability, and function of transduced T cells. We sought to test the importance of FBS and human FP in achieving efficient transduction of activated T cells, and documented that FP is a viable alternative to the use of bovine products during the transduction period. It appears that the processing of the plasma as described removes complement or other agents that have been described to interfere retroviral mediated gene transfer (Shimizu *et al.*, 1995). The role of IL-2 in the optimization of the expansion and subsequent function of transduced and selected T cells remains unclear. Current investigations are proceeding in our laboratory to determine if IL-2 can be limited or eliminated, and how this effects transduction, expansion, and the ability of T cells to respond to a second stimulus.

In summary, we describe a successful preclinical protocol in which activation of human T cells is achieved with anti-CD3/CD28 beads, facilitating efficient retroviral mediated gene transfer and selection of the transduced population using clinical scale magnetic beads devices.

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Use of anti-CD3/CD28 mAb coupled magnetic beads permitting subsequent phenotypic analysis of activated human T cells by indirect immunofluorescence

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Abstract

Functional analysis of T lymphocytes requires in vitro stimulation of these cells under experimental conditions that mimic as closely as possible physiological in vivo stimulation and that involve antigen/T cell receptor (TCR)-mediated activation. Because of the low frequency of antigen-specific T cells in human clinical samples, stimulation with a combination of anti-CD3 and anti-CD28 monoclonal antibodies (mAbs) is a preferred method. Interaction of these mAbs with their ligand results in modulation of the mAb–ligand complex from the cell surface. However, as a result of incomplete modulation, CD3/CD28 mAb complexes often remain at the cell surface, thereby precluding subsequent indirect immunofluorescence and flow cytometry analysis using mouse immunoglobulin (Ig)-specific antibodies. This is of importance in situations in which no specific fluorochrome-conjugated mAbs are available, such as in screening procedures of Ig-containing hybridoma culture supernatants. We propose here the use of CD3/CD28 mAbs, linked to magnetic beads allowing standardization of the activation conditions, optimal activation of T cells and complete modulation of antigen–antibody complexes from the cell surface.

Keywords: T lymphocytes; Cellular activation; Monoclonal antibodies; Magnetic beads; Immunofluorescence; FACS; Cytokine production

1. Introduction

Optimal activation of human T cells in vivo is ensured by the interaction of specific antigen with the T cell receptor (TCR) in the presence of co-stimulatory signals, provided for by antigen-presenting cells (APC). As the frequency of human antigen-specific T cells in peripheral blood mononuclear cell samples is very low and autologous or HLA-matched APC are rarely available, stimulation of T cells in vitro is often carried out with polyclonal activators such as the lectins PHA or Con A or with the combination of the phorbol ester TPA and calcium ionophore. Although these modes of activation may provide useful information on signalling pathways in T cells, they are, from a biological point of view,

Abbreviations: mAb, monoclonal antibody; Ig, immunoglobulin; TCR, T cell receptor; APC, antigen-presenting cells.

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artificial and not always comparable to in vivo stimulation conditions. For example, cytokine production profiles of human T cells, following stimulation with TPA and phorbol ester stimulation, differ from those that result from stimulation with specific antigen and APC (Yssel et al., 1992; Byun et al., 1994; Yang et al., 1995). A more physiologically relevant mode of activation is stimulation of T cells with a combination of a monoclonal antibody (mAb) specific for CD3, which mimics antigen-mediated T cell receptor (TCR)/CD3 complex-mediated signalling (Reinherz et al., 1982), and an anti-CD28 mAb which provides the required co-stimulatory signals (Hara et al., 1985; Geppert and Lipsky, 1987; Schwartz, 1990). Optimal stimulation of highly purified T cells requires the presence of accessory cells (Davis et al., 1986). However, this requirement is bypassed by the use of multivalent anti-CD3 mAb, either cross-linked onto tissue culture plates (Geppert and Lipsky, 1987), covalently linked to Sepharose beads, or in combination with an anti-mouse immunoglobulin (Ig)G (Umetsu et al., 1987), which results in cross-linking of CD3 on the cell surface. In contrast, triggering of CD28 does not require such cross-linking (Hara et al., 1985) and T cell activation is carried out with soluble mAbs (Levine et al., 1996, 1997).

Interaction of CD3 with a specific mAb results in its down-regulation and subsequent internalization of the CD3–mAb complex (Antel et al., 1982; Reinherz et al., 1982; Telerman et al., 1987). However, total loss of cell surface protein/mAb expression is dependent on optimal experimental conditions in vitro, as well as on the reagents used, and often, due to partial modulation, such complexes remain at the cell surface (see present study). The remainder of these protein/mAb complexes will interfere with subsequent immunostaining of cell surface molecules expressed on these cells, for which no directly conjugated mAbs are available.

In the present study, we have analyzed whether the use of mAbs linked to magnetic beads results in complete modulation of CD3/CD28 mAb complexes from the surface of T cells, thus permitting staining of other cell surface molecules expressed on these cells by indirect immunofluorescence and flow cytometry.

2. Materials and methods

2.1. Cells and culture conditions

Mononuclear cells were isolated from freshly collected peripheral blood from healthy individuals (Etablissement Français du Sang, Montpellier, France) by Ficoll-Hypaque density gradient centrifugation. $CD4^+$ T cells were purified (purity >95%) by negative selection from mononuclear cell preparations, using the RosetteSep procedure (StemCell Technologies, Vancouver, Canada), according to the manufacturer's instructions. The $CD4^+$ T cell clones used in this study were generated (Lecart et al., 2001) and maintained in culture (Yssel and Spits, 2002) as described previously.

To carry out a meaningful comparison between the different experimental conditions, the same two mAbs were used throughout the study: T cells (2×10^6 cells/ml) were stimulated with the anti-CD3 mAb SPV-T3b (IgG2a, Spits et al., 1983) and the anti-CD28 mAb L293 (IgG1, Testi and Lanier, 1989). Two modes of activation were used: stimulation of T cells with both mAbs, covalently linked onto magnetic beads (Expander Beads, Dynal, Oslo, Norway), was compared with a stimulation involving anti-CD3 mAb immobilized onto plastic tissue culture plates and soluble (1 μg/ml) anti-CD28 mAb (Umetsu et al., 1987; Levine et al., 1996). For coating onto plastic, anti-CD3 mAb was incubated in 24-well tissue culture plates (Nunc, Roskilde, Denmark) at a concentration of 10 μg/ml in PBS. After incubation at 4 °C for 24 h, the wells were washed twice with PBS and once with culture medium prior to the addition of the cells. When using plate-bound anti-CD3 mAb for stimulation, plates were spun for 2 min at $190 \times g$ prior to incubation in order to enhance interaction of the cells with the mAb (Yssel and Spits, 2002). T cells were harvested after incubation for 24 h at 37 °C and 5% CO_2 for analysis by immunofluorescence and flow cytometry. Culture supernatants were harvested after 48 h of incubation for analysis by ELISA. Cultures of T cells that had been stimulated with bead-linked mAbs were passed over a magnetic particle separator prior to analysis in order to remove magnetic beads (see under Section 2.3). All experiments were carried out in Yssel's medium (Yssel et al., 1984), supplemented with 1% human AB^+ serum (Etablissement Français du Sang, Lyon, France).

2.2. Immunofluorescence and flow cytometry

All immunofluorescence and flow cytometry procedures were carried out as described (Scheffold et al., 2001). The following (m)Abs were used: FITC-conjugated and non-conjugated anti-CD3 mAb B-B10 and anti-CD25 mAb (kindly provided by Dr. John Wijdenes, Diaclone Research, Besançon, France), a non-conjugated anti-CD69 mAb, a non-conjugated and FITC-conjugated IgG1 mAb as isotype-specific negative controls (purchased from Becton-Dickinson, San Jose, CA) and a FITC-labelled goat anti-mouse Ig Ab (Caltag, Burlingame, CA). Results were analyzed on a FACSCalibur® flow cytometer, calibrated with FITC-coupled Callibrite® beads according to the recommendations of the manufacturer and equipped with Cellquest software (Becton Dickinson). Settings of the flow cytometer for a typical experiment were as follows: Detector FSC, voltage E00, AmpGain 2.25, Mode Lin; Detector FCS: voltage E00, AmpGain 2.25, Mode Lin; Detector SSC, voltage 577, AmpGain 1.12, Mode Lin; Detector FL1: voltage 752, AmpGain 1.00, Mode Log.

2.3. Detection and removal of shed CD3/CD28 mAb complexes

The presence of shed CD3/CD28 mAb complexes in culture supernatants was measured by ELISA as follows: wells of a 96-well flat bottom plate were coated with a goat anti-mouse IgG (γ and L chain-specific) Ab (Caltech) at a concentration of 2 μg/ml for 2 h at 37 °C and washed with PBS, supplemented with 0.05% Tween. Culture supernatants to be tested were incubated for 1.5 h at room temperature and after washing, a goat anti-mouse Ig coupled to alkaline phosphatase (Dako, Trappes, France) was added at a dilution of 1:1000. After an incubation of 1 h at room temperature, wells were washed, substrate (Sigma 104, Sigma-Aldrich, L'isle d'Abeau Chesnes, France) was added and the absorbance measured at 450 nm in an ELISA plate reader (Dynatech, MR5000). As a standard, mouse IgG (Southern) was used. The detection limit of the assay was 5 ng/ml.

Shed bead-linked CD3/CD28 Ab complexes were removed by incubating culture supernatants with goat anti-mouse Ig-coated magnetic beads (Dynal) at 4 °C for 30 min in 2-ml vials on a rotating device, followed by placing the vials in a magnetic particle separator (Miltenyi Biotech, Paris, France). Culture supernatants from T cells stimulated with plate-bound mAbs were incubated with goat anti-mouse Ig linked magnetic beads (Dynal) and further treated as described above.

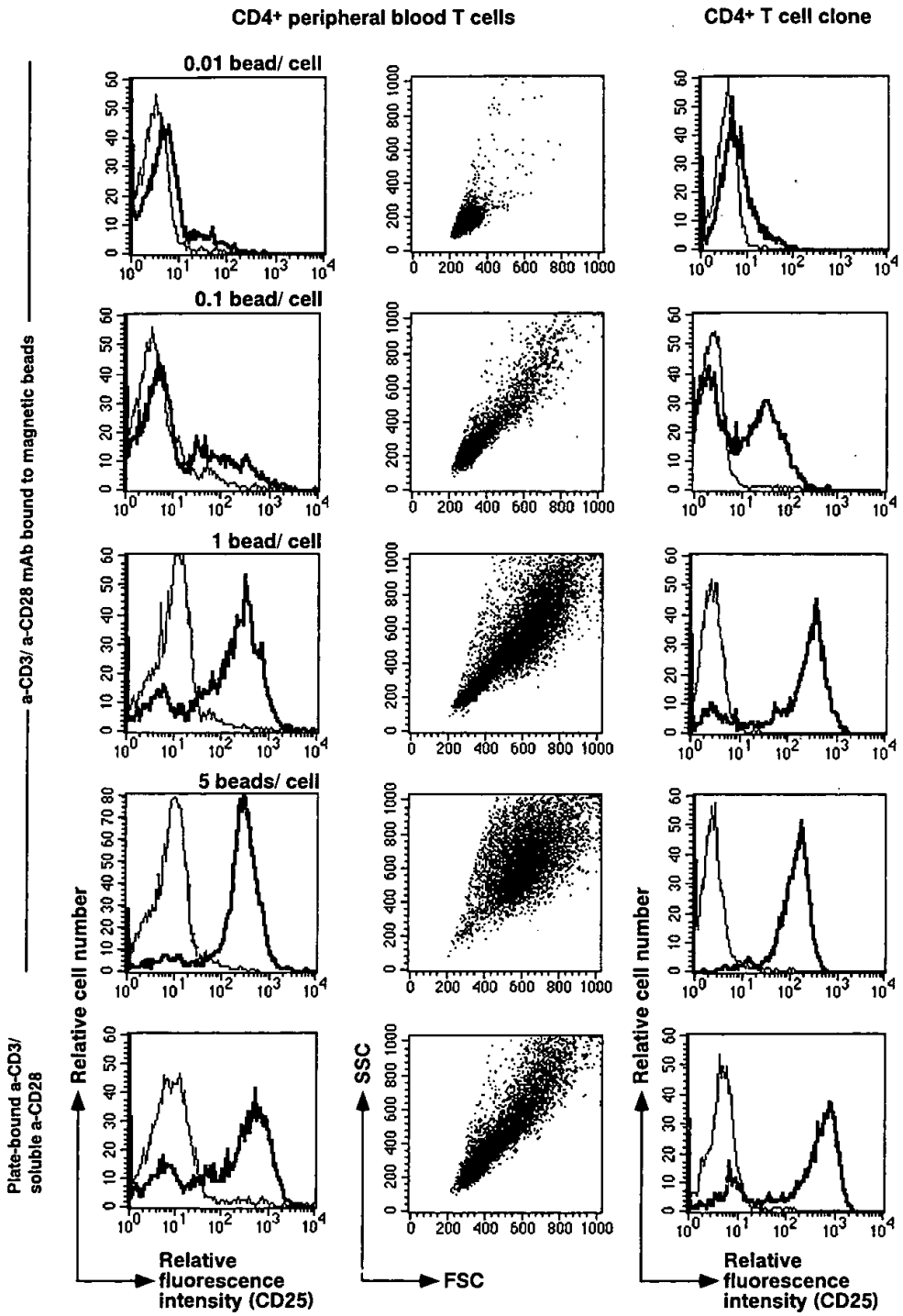
2.4. Analysis of cytokine production by ELISA

Two-hundred-thousand cloned human T cells were transferred to the flat bottom wells of a 96-well culture plate (Nunc) and stimulated as described under Section 2.1. After incubation of the cultures at 37 °C 5% CO_2 for 48 h, production of IL-4, IL-5, IL-10 and IFN-γ was analyzed by specific ELISA, as described previously (Pène et al., 1998; Lecart et al., 2001).

3. Results and discussion

3.1. Magnetic bead-linked and plate-bound anti-CD3/soluble anti-CD28 mAbs activate $CD4^+$ T cells to a similar extent

The concentration of magnetic bead-linked anti-CD3 and anti-CD28 mAbs required to induce optimal T cell activation was determined by analyzing the expression of CD25, as expression levels of this cell surface molecule correlate with the magnitude of activation. Maximal T cell activation was observed at a concentration of about 5 magnetic beads/cell, resulting in high levels of CD25 expression on more than 90% of purified $CD4^+$ T cells (Fig. 1). A bead/T cell ration of 10:1 yielded similar results (data not shown; Anne-Marie-Rasmussen, Dynal Biotech, Oslo, unpublished results). The frequency of $CD25^+$ T cells stimulated with bead-linked mAbs was generally somewhat higher as compared to that following stimulation with plate-bound anti-CD3/soluble anti-CD28 mAbs (Fig. 1). Furthermore, cytometric analysis showed that the magnitude of activation following the different stimulation conditions also correlated with the side scatter (SSC) and forward scatter (FSC) properties, representing granularity and size, respectively, of the cells. Similar results were obtained with $CD4^+$ T cell clones (Fig. 1 and other results not shown).


CD4+ peripheral blood T cells
CD4+ T cell clone
0.01 bead/ cell
0.1 bead/ cell
1 bead/ cell
5 beads/ cell
a-CD3/ a-CD28 mAb bound to magnetic beads
Plate-bound a-CD3/ soluble a-CD28
Relative cell number
Relative fluorescence intensity (CD25)
SSC
FSC

The capacity of both modes of activation to induce the production of cytokines was also tested on $CD4^+$ T cell clones. Results from experiments with two T cell clones with a Th2-like cytokine production profile, permitting the analysis of several cytokines, are shown in Table 1. Similar to the results with respect to CD25 expression, levels of cytokine production correlated with the amounts of magnetic bead-linked mAbs used to stimulate the cells, with a plateau of about 5 beads/cell. Stimulation of the cells with a higher bead-to-cell ratio generally resulted in somewhat better sustained, although not statistically significant, cytokine production levels (results not shown). Cytokine production levels were comparable to those following stimulation with plate-bound anti-CD3/soluble anti-CD28 mAbs. Moreover, cytokine production profiles did not differ between both stimulation conditions. Together, these results show that mAbs directed at CD3 and CD28, and covalently linked to magnetic beads, are able to induce appropriate T cell activation.

Table 1
Activation-inducing capacity of magnetic bead-linked anti-CD3/CD28 mAb, as compared to plate-bound anti-CD3/soluble anti-CD28 mAbs

Stimulation conditions	Cytokine production (ng/ml ± S.D.)[a]			
	IL-4	IL-5	IFN-γ	IL-10
BOY.JF157				
Medium	<0.05	<0.01	<0.01	<0.01
Immob-a-CD3/s a-CD28[b]	14.6 ± 3.9	9.5 ± 4.0	<0.01	5.9 ± 2.9
Bead-linked aCD3/CD28[c]				
0.01 bead/cell	1.5 ± 1.3	2.6 ± 1.3	<0.01	2.2 ± 1.2
0.1 bead/cell	7.5 ± 3.4	11.0 ± 5.8	<0.01	4.9 ± 1.8
1 bead/cell	10.1 ± 4.4	14.5 ± 4.1	<0.01	5.9 ± 2.8
5 beads/cell	12.7 ± 4.8	16.7 ± 3.4	<0.01	4.5 ± 2.4
PUE.F3.9				
Medium	<0.01	<0.01	<0.01	<0.01
Immob-a-CD3/s a-CD28[b]	6.4 ± 3.5	14.0 ± 4.1	4.0 ± 2.2	5.5 ± 1.9
bead-linked aCD3/CD28[c]				
0.01 bead/cell	1.4 ± 0.5	7.5 ± 2.2	0.3 ± 0.1	1.4 ± 0.2
0.1 bead/cell	5.8 ± 1.6	20.0 ± 3.5	1.8 ± 0.1	5.8 ± 1.3
1 bead/cell	8.2 ± 4.3	22.5 ± 4.0	4.3 ± 0.2	8.2 ± 2.6
5 beads/cell	9.8 ± 3.4	19.7 ± 5.1	3.7 ± 0.9	10.7 ± 4.8

[a] The Th2 T cell clone BOY.JF157 and the Th0/Th2 T cell clone PUEF.3.9 were stimulated with anti-CD3 from 3 independent experiments.

[b] Plate-bound SPV-T3b (10 μg/ml coating) and soluble L293 mAb (1 μg/ml).

[c] SPV-T3b and L293 mAbs cross-linked onto magnetic beads.

3.2. Stimulation of $CD4^+$ T cells with magnetic bead-linked anti-CD3 and anti-CD28 mAbs results in complete removal of CD3/CD28 mAb complexes from the cell surface

Interaction of certain cell surface molecules with specific mAbs may result in the down-regulation and shedding (or internalization) of the cell surface molecule/mAb complex (Antel et al., 1982; Reinherz et al., 1982, Telerman et al., 1987). We therefore determined levels of cell surface expression of CD3/CD28 mAb complexes on activated $CD4^+$ T cells following their stimulation with magnetic bead-linked mAbs or plate-bound anti-CD3/soluble anti-CD28 mAbs. Significant levels of such complexes were still present at the surface of T cells stimulated with plate-bound anti-CD3/soluble anti-CD28 mAbs, as detected by staining with an FITC-conjugated Ab specific for mouse Ig (Fig. 2A). In contrast, however, no CD3/CD28/mAb complex was detectable on T cells that had been stimulated with anti-CD3/CD28 mAb immobilized on magnetic beads, as shown in a representative experiment in Fig. 2B. From a methodological point of view, the absence of residual anti-CD3 and anti-CD28 mAbs on the cell surface permits subsequent staining of the T cells for the expression of other cell surface molecules by indirect immunofluorescence, as is shown for CD25 and CD69 in Fig. 2D.

Fig. 1. Expression of CD25 by $CD4^+$ peripheral blood T cells and T cell clones following stimulation with anti-CD3 and anti-CD28 mAbs. One million cells per milliliter of purified $CD4^+$ peripheral blood T cells or of Th2 cell clone BOY.JF157 were stimulated either with different concentrations of the anti-CD3 mAb SPV-T3b and anti-CD28 mAb L293, covalently linked to magnetic beads, or with SPV-T3b, immobilized to tissue culture plates and soluble L293, as described in Materials and methods. Expressions of CD25 and SSC (granularity)/FSC (size) were measured by immunofluorescence and/or flow cytometry. Histograms from cells stained with the FITC-conjugated anti-CD25 mAb B-B1 (bold histograms) are superimposed over those from an isotype-matched control mAb (thin histograms). The x and y axes represent fluorescence (four decade log scale) and relative cell number, respectively. Representative results from six independent experiments.

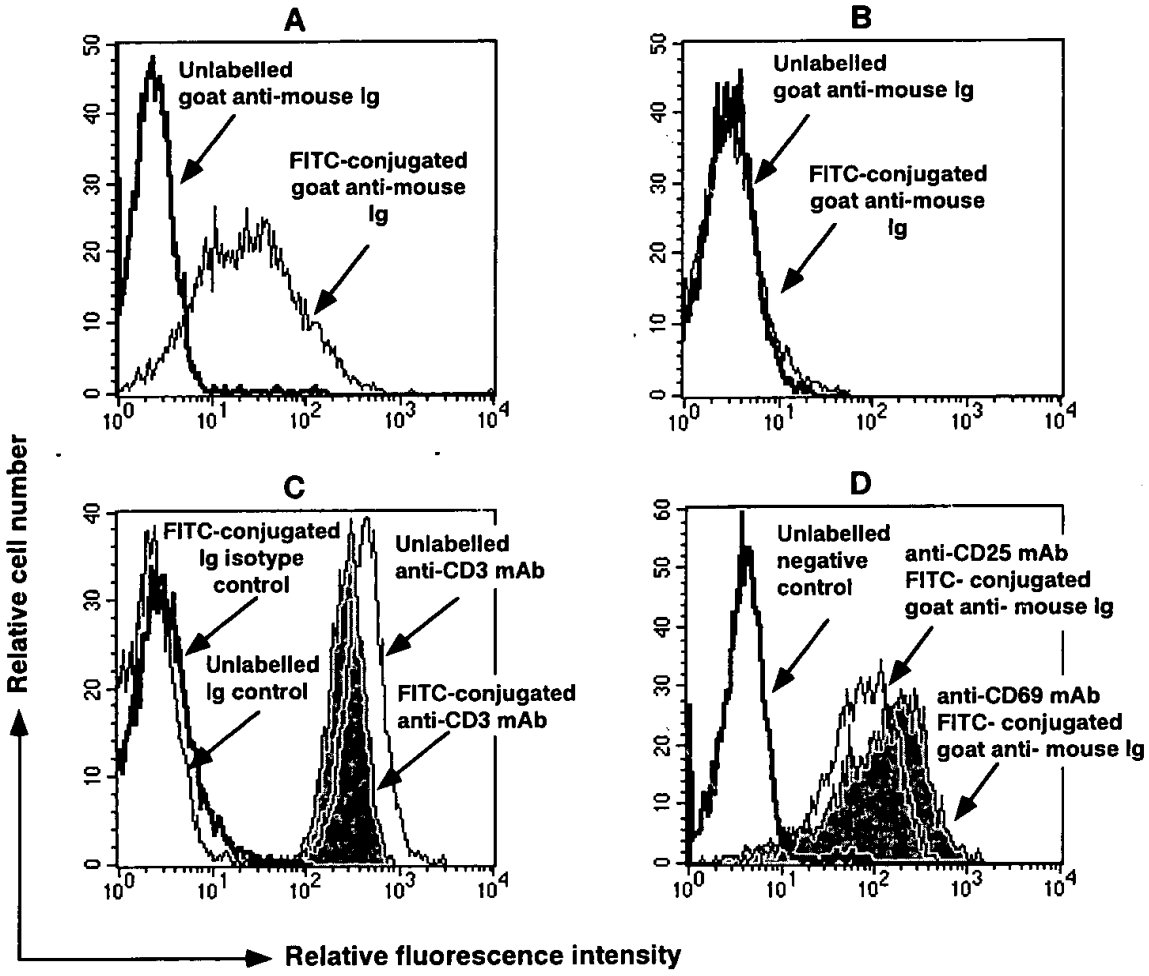


Fig. 2. Immunofluorescence staining pattern of a T cell clone with anti-CD3 and anti-CD28 mAbs. T cell clone BOY.JF157 (10^6 cells/ml) was stimulated either with plate-bound anti-CD3 and soluble anti-CD28 mAb (A) or with these mAbs linked onto magnetic beads (B, C, D). Staining conditions are indicated in the histograms. The x and y axes represent fluorescence (four decade log scale) and relative cell number, respectively. Representative results of two independent experiments.

The down-regulation of cell surface CD3, following binding of an anti-CD3 mAb (Antel et al., 1982; Reinherz et al., 1982), mimics the fate of the TCR following interaction with its physiologic ligand, since it has been shown by Valitutti et al. (1997) that TCR/CD3 complex expression is also down-regulated on T cells following stimulation with peptide-pulsed APC. This modulation from the cell surface is associated with an antigen dose-dependent decrease of the total cellular content of CD3-ε, and ζ chains. The time-course of the decrease in overall CD3 protein expression was found by these authors to overlap with the kinetics of TCR down-regulation and suggests a rapid degradation of internalized TCR–CD3 complexes (Valitutti et al., 1997).

It is of note that T cells, stimulated with anti-CD3 mAb, still expressed significant levels of CD3 as revealed by staining with a directly FITC-conjugated anti-CD3 mAb (Fig. 2C), indicating that only a fraction of the CD3 molecules, bound to the bead-linked anti-CD3 mAb, had been modulated from the cell surface. In the same vein, this observation also

shows that only a few anti-CD3 mAbs are required to activate the cells. Indeed, Valitutti et al. (1995) have reported that a very small number of TCR molecules is involved in the interaction with specific antigen and APC, thereby transducing an activation signal via CD3. Taken together, the results presented here support the physiologic relevance of stimulation of T cells with anti-CD3 mAb-bound beads, which constitutes an alternative for the use of specific antigen because of the cumbersome and technically very demanding procedure of large-scale culture of human antigen-specific T cell lines.

Monocytes are able to engulf the type of magnetic beads used in the present study (Anne-Marie-Rasmussen, Dynal Biotech, personal communication). However, due to their size and physical properties, these beads, unlike anti-CD3 mAb/protein complexes, are not taken up by T cells and the modulation of these complexes from the cell surface is likely due to shedding, rather than their internalization. Indeed, high levels of CD3-anti-CD3 mAb complexes were detected in culture supernatants from T cells stimulated with mAb-linked magnetic beads. However, these complexes were also present in supernatants from T cells stimulated with anti-CD3 mAb immobilized onto a plastic tissue culture plate. This is somewhat unexpected since only very low quantities of soluble mAb could be detected in the culture medium in these wells already after 18 h of incubation at 37 °C in the absence of T cells (Table 2), indicating that once incubated on a plastic surface, anti-CD3 mAb remains relatively firmly attached. It seems, therefore, that interaction with T cell surface-expressed CD3 will strip the mAb off the tissue culture plate and that the CD3/anti-CD3 mAb complex is subsequently shed from the cell surface. The presence of such complexes in culture supernatants might interfere with functional assays which are based on proliferative responses of T cells, activated via immobilized anti-CD3 and CD28 mAb, as a read-out system. Yet, complexes of cell surface molecules and mAbs can easily be removed using a goat-anti mouse Ab coupled to magnetic beads (Table 2).

A major problem with the use of mAbs immobilized on plastic is the lack of quantification and reproducibility of the various methods used to coat the mAbs. Moreover, a mAb will adhere to the plastic surface with both its F_c portion and its variable F_{ab} region, whereas over time a number of the mAbs will detach and become soluble (Table 2). In this respect, the use of mAbs, linked to magnetic beads, allows optimization and standardization of cellular activation conditions, thus enhancing the reproducibility of the experiments. Taken together, the data presented here show that stimulation of T cells with magnetic bead-linked anti-CD3 and anti-CD28 mAb provides a physiologic mode of activation which, due to its capacity to completely remove the CD3/CD28 molecules from the cell surface, permits further analysis of cell surface molecules expressed on these cells by indirect immunofluorescence and flow cytometry. Finally, we should like to stress that this method has been successfully applied for the screening of T cell subpopulation-specific Ig-containing hybridoma supernatants in our laboratory (J. Pène and H, Yssel, unpublished data).

Table 2
Interaction of CD3 with plate-bound anti-CD3 mAbs results in the shedding of anti-CD3/mAb complexes into the culture supernatant

Conditions[a]	CD3/mAb protein concentration (ng/ml)	
	Exp 1	Exp 2
Medium	12	<5
BOY.JF157	107	79
BOY.JF161	134	230
After removal with goat anti-mouse Ab[b]		
Medium	<5	<5
BOY.JF157	<5	<5
BOY.JF161	<5	<5

[a] Wells of a 24-well tissue culture plate were coated with anti-CD3 mAb, as described in Materials and methods and incubated with culture medium, with the $CD4^+$ Th2 clone BOY.JF157 or the Tr1 clone BOY.JF161 (2×10^6 cells/ml). After 18 h of incubation, culture supernatants were harvested and levels of soluble anti-CD3 mAb/CD3 complexes were determined by ELISA.

[b] Culture supernatants were incubated with an Ig-specific goat anti-mouse Ab, linked to magnetic beads and treated as described in Materials and methods.

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